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NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1977 through September 30, 1978

Part II-B

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

US National Cancer Institute
Report of Program Activities

SUMMARY REPORT
IMMUNOLOGY BRANCH
Fiscal Year 1978

The Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis given to studies of regulation and control of immune responses, structure and function of cell surfaces, transplantation biology and tumor immunology. In addition, clinical studies of relevance to tumor immunology are performed.

A. REGULATION AND CONTROL OF IMMUNE RESPONSES

It is known that specific antibody synthesis is controlled by genes located in or near the major histocompatibility complex (MHC). The nature and function of the genetic control of the cellular interactions involved in antibody responses are under investigation. An in vitro system for generating both primary and secondary antibody responses to soluble antigens has been developed. Responses in the system are both T cell and accessory cell dependent and, for TNP-TGAL and TNP-HGAL, are clearly under H-2 linked Ir gene control. The function of accessory cells in responses to TNP-TGAL and TNP-HGAL is also regulated by H-2 linked Ir genes and these are encoded within the same subregions of H-2 as is the overall in vitro and in vivo response to these antigens. The generation of specific antibody responses requires the active recognition by T cells of H-2 antigens (probably Ia antigens) as "self" markers that are expressed by accessory cells. Moreover, the H-2 antigens that are recognized as "self" are not determined by the genotype of the T cells, but rather by the genotype of the thymus in which the T cells mature (CB-05064).

Accessory cells which support antigen specific primed lymph node T cell proliferation are Thy 1.2 negative, radioresistant, glass adherent and express both I-A and I-E or C subregion antigens (CB-05065).

Genetic control is also being studied in vivo, utilizing the complex antigen staphylococcal nuclease. Antibody responses to this antigen are under control of H-2-linked immune response genes. Antibodies against idiotypic determinants on the anti-nuclease antibody have been produced in rats and used to screen a variety of strains for the presence of idiotype. Results of these studies indicate that idiotype expression is linked to the heavy chain allotype marker, rather than to immune response gene loci. A large recombination frequency (7-10%) was found, but the reason for this high recombination frequency is not known. Progeny testing proved that these were true recombinant animals. Both high responder and low responder animals (B10.A and B10, respectively) were found to make comparable levels of anti-nuclease antibody following multiple boosts with antigen. However, the proportion of antibody against the carboxy terminal third of the nuclease molecule was very different in these two strains. This indicates that H-2-linked Ir gene control must exert an effect at the B cell as well as at the T cell level (CB-05036).

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Viruses or chemicals can modify the surfaces of cells to create new antigens that depend both on the modifying agent and on the histocompatibility antigens of the cell surface being modified. TNP modified spleen cells induce cytotoxic effector cells which only lyse TNP modified target cells and only if those target cells share appropriate H-2 antigens with both the stimulating and the effector cells. Specificity of the effectors is such that they cannot lyse H-2 matched targets in which the TNP group is presented on the targets by insertion of a TNP-fatty acid dextran conjugate into the cell membrane. Effector cells can be generated, however, by culturing spleen cells with TNP coupled to soluble proteins. Further studies indicate that phagocytic accessory cells are required for generating cytotoxic cells, interaction of TNP-protein with cell surfaces is not mediated by a phagocytic mechanism and H-2 restriction is not determined by the phagocytic accessory cells.

The ability to generate TNP-dependent cytotoxicity was altered by injecting mice with trinitrobenzene sulfonic acid (TNBS). Short term priming was followed by specific unresponsiveness four months later (CB-05038). In this same system, studies of the generation of antibody forming cells were carried out utilizing a plaque forming cell assay with TNP-modified sheep erythrocytes as targets. Plaque forming cells could be generated in some strains but not in others even though all of the tested strains generated strong cytotoxic cellular responses against TNP modified self cells. This indicates that the antibody response against TNP coupled to syngeneic cells is controlled by genetic factors mapping within the H-2 complex which do not affect T cell mediated cytotoxicity against TNP-self structures, and suggests that these antibodies and T cell receptors recognize different antigenic specificities (CB-05066).

Studies within this system have been extended to determining the ability of human peripheral blood leukocytes to be stimulated in vitro against TNP-modified autologous leukocytes. Proliferative and cytotoxic responses were obtained and the results indicated that the T cells involved did not recognize TNP exclusively but rather TNP in association with human-specific self determinants. These determinants seem to include ones that are polymorphic and associated with serologically defined HLA-A and -B locus antigens, those that are polymorphic and HLA linked but not strongly associated with serologically determined antigens, and "public" determinants with little if any polymorphism detected. These results suggest that human T cells recognize a variety of self determinants in association with TNP (CB-05067).

Conditions have been developed for in vitro stimulation of human peripheral blood leukocyte effector cells cytotoxic for influenza infected target cells and for measurement of effector cell activity on virus infected cryopreserved human peripheral blood leukocyte targets. The use of a variety of influenza viruses has led to results suggesting that human T cell responses to influenza viruses may be type-specific and that they also appear to be restricted to recognize self components (CB-05078).

The phenomenon of in vitro auto-sensitization has been studied further and it was shown that effectors preferentially lysed H-2 matched targets. Specificity studies suggested that effector cells were sensitized against components contained in fetal calf serum, but not in horse serum. Enhanced responses were obtained when mice were preinjected with irradiated syngeneic cells raising the possibility that the response is dependent on natural killer cells (CB-05077).

Functional and serologic characterization of cells important to the immune response have continued. The expression of Ia antigens on functional cell subpopulations has been studied. Anti-Ia plus complement has been shown to inactivate a glass adherent non-T, non-B radio-resistant accessory cell which is required for the induction of a proliferative response to concanavallin A. Genetic studies have shown that the Ia determinants involved are encoded by at least two different subregions of the I locus. Elimination of these accessory cells also seems to limit generation of cell mediated lympholysis suppressor cells of the type induced by five-day culturing of normal spleen cells. These accessory cells appear to be the same cells as those required for the in vitro generation of T cell dependent IgM responses to soluble TNP-conjugated protein antigens (reported under Project CB-05064) (CB-05069).

Similarities have been observed between F_1 anti-parent marrow graft rejection in vivo and the development of F_1 anti-parent cytotoxic of effector cells in vitro. A number of differences have also been noted:

1. The in vivo rejection process does not require active immunization while the in vitro development of cytotoxic effectors does;

2. Marrow graft rejection is neither caused by nor dependent upon thymus derived cells, while the F_1 anti-parent effectors and their precursors are T cells;

3. The recognition of parental structures by F_1 cells in the F_1 anti-parent graft rejection system appears to involve determinants coded for by a gene mapping near H-2D, whereas for F_1 anti-parent cytotoxicity, products of additional genes within the H-2 complex are also important. Comparisons of natural killer cell activity in vitro, F_1 anti-parent cytotoxicity in in vitro and F_1 anti-parent marrow graft rejection in vitro indicate that all three phenomena probably share a common cell which may serve in an accessory role for each type of response (CB-05049).

Both cellular and humoral regulators of immune responses are under investi gation. An Ia positive accessory cell is required for the generation of suppressor cells in vitro. The suppressor cells themselves, however, are resistant to anti-Ia plus complement. Mouse strains differ in their ability to generate culture-induced suppressor cells. Certain strains do not demonstrate suppressor activity and the defect appears to be in the ability to generate suppressor cells rather than in the susceptibility of target cells to suppresion (CB-05055). The potential role of idiotype bearing molecules in the regulation of immune responses is also being studied. T lymphocytes express anti-TGAL idiotypes identical to or cross reactive with those found in anti-TGAL antibodies produced in the same mouse strain. The functional implications of these observations remain to be determined (CB-05058). Antigen specific T cell factors have been shown to express immunoregulatory functions in vivo but preliminary experiments have not provided evidence for activity in specific B cell activation or T-B cooperation in vitro (CB-05059).

B. STRUCTURE AND FUNCTION OF CELL SURFACES

Synthetic membrane antigens have been created and compared with natural membrane antigens of the same cells in an attempt to explore capping and other other phenomena. Lateral diffusion constants for these synthetic macromolecules have been determined and shown to be typical of membrane proteins. Addition of antibody to these dextran antigens causes patching and capping with a consequent reduction in lateral diffusion. With very flat fibroblasts, these adsorbed synthetic membrane molecules form patches in rows similar to those that have been observed for natural membrane antigens. Thymocytes adsorb these synthetic antigens from solution more readily than do splenocytes and cap the adsorbed molecules more slowly than do splenocytes. Drug inhibition of capping of adsorbed molecules on lymphoid cells showed characteristics similar to those seen with capping natural markers such as surface immunoglobulin (CB-05068).

The nature of cell membrane damage caused by immunologic mechanisms is under investigation. Damage caused by antibody plus complement is associated with complete loss of membrane potential and electrical resistance as determined by intracellular recording performed with microelectrodes. These changes are noted within 1-2 minutes of the addition of complement to the system but are reversible if complement is removed from the system. Additional information concerning membrane lesions has been obtained using resealed red blood cell ghost membranes. These studies indicate that the lesions caused by complement plus antibody are of such a size that they will allow materials of 40,000 molecular weight to pass through the membrane but will not allow molecules of 80,000 molecular weight to do so. Using the same basic techniques, studies with antibody dependent cell mediated cytotoxicity shows that molecules of 500,000 molecular weight can pass through the membrane but molecules of 2,000,000 molecular weight cannot. It thus appears that there is a discrete size pore created in the membrane after damage created either by antibody plus complement or antibody plus appropriate attacker cells (CB-05018).

The interaction of complement components with immunoglobulins and cell surfaces is under investigation. The ability of IgG to initiate complement dependent lysis appears to increase gradually with the size of the IgG complex. In contrast, cell mediated lysis (antibody dependent cell mediated cytotoxicity) of oligomer-coated cells appears to be independent of the size of the complex and depends only on the number of IgG molecules bound per cell (CB-05050).

Continued attempts to isolate Fc receptors from detergent solubilized murine lymphoid cells have been unsuccessful (CB-05048).

A number of studies concerning the binding properties of Fc receptors have been carried out. With human peripheral lymphocytes, it has been shown that the Fc receptors of B lymphocytes and UL-cells differ in their avidity for immunoglobulin complexes. In addition, the Fc receptors of UL-cells do not appear to be associated with Ia-like antigens (CB-05034).

When oligomers of IgG were studied, it was shown that although the number of receptors for IgG varied between cell populations, the affinities of each cell type are roughly the same (CB-05050).

In the mouse, Fc receptors of B cells and T cells, but not UL cells or macrophages, appear to be associated with Ia antigens on the cell surface. In the case of the B cell, Fc receptors and Ia antigens appear to be distinct molecules and the association is ligand induced (CB-05035).

Studies have attempted to define functional consequences of interactions with the Fc receptor. The intracellular levels of cAMP were consistently elevated after 30 minutes of contact between mouse spleen cells and surface bound antigen antibody complexes. No significant change in cGMP levels was found under the same experimental conditions (CB-05060). The capping of Fc receptors on murine B cells was markedly inhibited by a combination of cytochalasin B and cochalasin suggesting that Fc receptors of B lymphocytes may be linked to lymphocyte cytoskeleton (CB-05035).

C. TRANSPLANTATION

Studies are being directed towards an understanding of the role of the products of the major histocompatibility complex in the cellular interactions of the immune response. Ia antigens have been shown to be associated with or responsible for mixed lymphocyte culture reactions. Antibodies to distinct Ia antigens have been shown to specifically inhibit the MLC reaction, apparently by blocking the stimulator cell (CB-05021). Cytotoxicity tests and absorption analyses indicate the presence of a previously unreported subregion within the Ir region of the H-2 complex and this subregion has been given the name I-E (CB-05021). Immune precipitation reactions have been used to isolate Ia-like antigens from rat lymphoid cells and these have been shown to be of approximately the same size as mouse Ia antigens (CB-05021).

Cell surface antigens associated with the Fc receptor have been detected. A single polymorphic non-H-2 locus, which determines at least four distinct antigens, has been detected and in one haplotype shown to be linked to the M-locus (CB-05035). A previously unrecognized H-2 locus within the D region has been demonstrated and named H-2L. A mutant mouse, the BALB/c-H-2^{db} has been studied and found to lack all specificities associated with the L molecule (CB-05021). The L alloantigens are capable of inducing allogeneic cytotoxic effector cells but they appear to be incapable of eliciting syngeneic effector cells specific for TNP modified antigens (CB-05076). In addition, an antibody response to a previously unrecognized H-2 antigen has been found and shown to be genetically controlled by a non-H-2-linked locus (CB-05021).

Three herds of miniature swine each homozygous for a different set of histocompatibility antigens at the major histocompatibility complex have been developed. Renal allografts within one of the homozygous herds appear to survive indefinitely despite minor histocompatibility antigenic differences.

However, rejection occurs following allografts within the other two herds, with variable kidney survival times (CB-05023). Cross immunizations between representatives of the three herds have led to the production of antisera apparently directed against antigens controlled by the major histocompatibility complex. These antisera have precipitated material from detergent solubilized cell surface preparations and by both size criteria and genetic criteria, the precipitated materials appear to be the precise homologs of murine H-2 and Ia anitgens (CB-05023).

Further studies of the histocompatibility antigens of Rhesus monkeys have led to the development of reagents recognizing three additional defined antigens. The RhLa system therefore now consists of 12 antigens in the A series and 12 in the B series. Some of these antisera appear to be detecting antigens analogous to Ia antigens (CB-05011). Antigens associated with the major histocompatibility complex of man have been associated with a variety of diseases. At diagnosis there appears to be an abnormal distribution of HLA antigens in patients with acute leukemia, with HLA-A2 being significantly elevated. A similar elevation of HLA-A2 in acute schizophrenia has also been noted (CB-05017).

Ia like antigens have been detected on human lymphoid cells using antisera derived from selected human donors. These antisera have been used to study the distribution of antigenic determinants in patients with the Sicca syndrome, as well as in patients with systemic lupus erythematosus. Certain antigens appear to be detected in higher frequency in these diseased populations than in individuals in the normal population. These antigens are also being studied in the Pima Indian population, a group of interest because of the high incidence of diabetes mellitus (CB-05006). Mouse alloantisera specific for murine Ia antigens appear to also react with human Ia-like antigens. Reciprocal reactivity between human antisera and murine Ia antigens was not detected (CB-05006).

Quantities of HLA gene products as well as of human Ia like antigens have been isolated from human lymphoid tissue culture cell lines and partially purified. In collaboration with Dr. Jack Strominger, these materials have been further purified and structurally analyzed (CB-05006).

Liposomes bearing hapten on their surface, have been used to quantitate cell surface receptors present on myeloma cells synthesizing immunoglobulin with hapten specificity (CB-05075).

D. TUMOR IMMUNOLOGY

The AKR mouse has been used in a number of studies of relevance to tumor immunology. Two different substrains of AKR, the AKR/J and the AKR/Cu have been investigated. AKR/Cu mice are known to have a mutation of chromosome 9 changing them from Thy 1.1 to Thy 1.2. Tumor lines have been derived from each substrain and, unexpectedly, reciprocal resistance has been demonstrated. Because of this, reciprocal CML studies have been performed. The results suggest that these two substrain also differ in the expression of a minor H antigen, linked to the Thy locus, which had never previously been recognized

(CB-05053). Several unusual AKR lymphoma lines which appear to exhibit characteristics of both T and B cells, have been studied. It remains unclear as to whether these tumor cells express alternative forms of differentiation antigens depending on whether they are growing in the spleen or lymph nodes, or whether the tumor itself consists of two subpopulations with different growth requirements and characteristics (CB-05043).

The antigenic characteristics of an AKR carcinoma have been studied. It appears that in this cell line there has been a loss of the normal AKR/J $H\text{-}2D^k$ private specificity along with a concomitant gain of an $H\text{-}2D^d$ specificity (CB-05051).

A number of primary methycholanthrene induced sarcomas, as well as tissue culture lines derived from them, have been analyzed for the presence of murine leukemia virus associated antigens. Many of the tumors react with fluoresceinated anti-MuLV antisera even though they are not producing intact virions as determined by GP70/P30 ratios or by culture for infectious MuLV. The fluorescence reactions are quite weak and can be detected only in the fluorescent activated cell sorter (CB-05003).

Spleen cells from mice bearing primary methylcholanthrene induced sarcomas are capable of generating normal primary cytotoxic cellular immune responses in vitro when a range of responder to stimulator cell ratios are studied. At certain ratios, abnormally low immune responses are detected but at other ratios, the same animals give normal results. This indicates that if there is any significant immunosuppression in animals bearing primary tumors, this suppression does not equally affect all aspects of in vitro function. By contrast, methylcholanthrene induced tumor cell lines are consistently immunosuppressive when cocultured in vitro with normal spleen cells and immunogen. Generation of cytotoxic cells against alloantigens, TNP-modified self and syngeneic tumors is blocked (CB-05003).

A randomized controlled clinical trial in human malignant melanoma is in progress. The patients are randomly assigned to receive treatment either with methyl CCNU, BCG alone or BCG plus allogeneic tissue culture grown vaccine. A fourth group consists of control patients who receive no further active treatment. The trial is still too early to permit evaluation but no striking differences between the groups have been noted to date (CB-05033). Studies of the peripheral blood lymphocytes of patients in this clinical trial indicated that greater than 90% of those receiving the cell vaccine generated cellular and/or humoral immune responses against one or more of the vaccine cell lines. In addition, all patients in the study had a normal capacity for generating cytotoxic cellular responses in vitro except for those patients receiving chemotherapy who showed some depression in this functional capacity. The use of BCG did not appear to increase the average in vitro natural killer or K cell cytotoxic immune activities (CB-05016). The psychosocial adjustment of these patients is also being studied and preliminary results suggest that there may be a psychological variable that is as useful as some of the other biological factors currently utilized in the prediction of short term prognosis for this malignancy (CB-05052).

E. FLUORESCENT ACTIVATED CELL SORTER FACILITY

The Immunology Branch continues to operate a Fluorescent Activated Cell Sorter which serves as a resource and has been utilized in a large number of collaborative investigations. This facility has been used to study expression of I and Ly antigens on thymocytes, the expression of cell surface antigens coated by xenotropic murine leukemia virus genomes on tumor cells, the characterization of liposome-cell interactions and early B-cell abnormalities in autoimmune mice. Details of these projects are reported in the appropriate annual reports.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05003-13 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell Mediated Cytotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
OTHER:	C. I. Civin	Clinical Associate	I NCI
	N. A. Dorfman	Visiting Fellow	I NCI
	R. I. Fisher	Senior Investigator	M NCI

COOPERATING UNITS (if any)

R. Lerner, Scripps Clinic and Research Foundation; C. K. Hsu, Frederick Cancer Research Center

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Mice bearing primary MCA-induced sarcomas are capable of generating normal or near normal cytotoxic cellular immune responses, using as a test primary in vitro spleen cell sensitization. Tumor-related immunosuppression does occur when irradiated tumor cells are cocultured with spleen cells during in vitro sensitization. This immunosuppression can be reversed by chemical modification of tumor cells, which also increases in vitro tumor immunogenicity.

2) Primary MCA-induced murine tumor cells have been adapted to rapid flow microfluorometry analysis. This technique has revealed that primary MCA-induced tumor cells stain with fluoresceinated anti-MuLV antisera at intensities undetected by routine fluorescent microscopy. Transfer of tumor cells to tissue culture growth conditions results in marked increases in anti-MuLV staining.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: This project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity in both humans and mice. Long-range goals are: (1) to identify and characterize factors which influence induction, maturation, and expression of cytotoxic cellular immune responses; (2) to define changes in cytotoxic cellular immunity which occur in tumor bearing hosts; and (3) to find means of manipulating the cytotoxic cellular immune response for therapeutic purposes. Immediate objectives of the past year have been to concentrate on goals #2 and #3.

Methods Employed: Cell-mediated cytotoxicity is measured in vitro by release of ^{51}Cr from labelled target cells incubated with lymphoid cells for 2-6 hours. Immune cells are collected from spleen, marrow, lymph node, thymus and peripheral blood of sensitized hosts. Several techniques have been used for fractionating cells. Peripheral blood cells (30-40 mls whole blood) and spleen cells (mouse) have been fractionated with Ficoll-Hypaque gradient centrifugation and/or on nylon columns; effluent cells from the nylon columns are highly enriched for T lymphocytes. In addition, immune cells have been produced by in vitro sensitization of spleen and peripheral blood cells using previously established Mishell and Dutton tissue culture techniques. Target cells are provided by freshly explanted cells, tissue culture lines, and established tumor passed in vivo or in vitro; most target cells function well if previously frozen in liquid nitrogen. Target cells have also been chemically modified in order to change the immunogenicity of cell surface antigens.

Sarcomas have been induced with methylcholanthrene (MCA) in mice from each of four inbred strains (NFS, C57BL/6N, BALB/c, and DBA/2), which are routinely screened for potentially immunosuppressive pathogens before admission to the colony at Frederick Cancer Research Center. Tumor cell lines established from these sarcomas are also screened for potentially immunosuppressive pathogens: mycoplasma (culture and serology) and a variety of viruses (serology) including MVM, Sendai, LDH and LCM. Both freshly explanted tumor fragments and early passaged cell lines have been frozen in liquid nitrogen.

Tumor-related nonspecific immunosuppression has been assessed by determining the capacity of spleen cells to generate cytotoxic cells in vitro against alloantigens, TNP-modified self and syngeneic MCA-induced sarcomas. Responses of spleen cells from tumor bearing hosts are compared with responses of cells from age-sex-strain-cage matched normal donors; in some experiments irradiated tumor cells are cocultured directly with normal spleen cells being sensitized in vitro. Data analysis includes variability in viable cell recoveries from cultures and from donor spleens.

Rapid flow microfluorometry has been adapted to analysis of sarcoma cells by preparing viable single cell suspensions from in situ tumors and normal tissues (collagenase and DNase ± trypsin) and from tumor and control cell lines (EDTA). Cells have been stained with $(Fab')_2$ fluoresceinated preparations of anti-MuLV generated against ecotropic Rauscher leukemia virus from

BALB/c 3T3 cells and xenotropic virus-infected SIRC cells. Similarly fractionated antisera with other specificities have been routinely used as controls.

Major Findings:

1) Spleen cells from mice bearing primary MCA induced leg sarcomas, approximately 1-2 centimeters in diameter, are capable of generating normal or near normal primary cytotoxic cellular immune responses in vitro. Responses have been generated against alloantigens, TNP-modified self and syngeneic sarcomas using 25 tumor bearing mice from C57BL/6, BALB/c and NFS strains. Some zones of responder/stimulator cell ratios show abnormally low immune responses with spleen cells from tumor bearing hosts, but responses of other zones in the same experiments have been normal or near normal. The point of these findings is that primary cytotoxic cellular immune responses can be generated in vitro with lymphoid cells from primary tumor bearing hosts without prefractionation of responder cells.

2) Spleen cells from normal C57BL/6N mice injected with either of two syngeneic MCA-induced tissue culture tumor lines have also been tested for their capacity to generate cytotoxic cells in vitro. Spleen cells from donors bearing tumors of approximately 1 centimeter in diameter are still capable of generating normal cytotoxic cell responses in vitro. To reconcile these findings with our earlier study of an MCA induced tumor line which was immunosuppressive in an identical test system, we suspect that tumor cell lines have inherently different potentials for immunosuppression or that the early studied tumor line contained an unknown immunosuppressive contaminant.

3) Each of three C57BL/6N methylcholanthrene induced tumor cell lines has been nonselectively immunosuppressive when cocultured with normal spleen cells and immunogen. Generation of cytotoxic cells against alloantigens, TNP-modified self and syngeneic tumors is blocked. Successful in vitro generation of cytotoxic cells occurs if tumor cells are treated with either formalin or tetranitromethane prior to coculture with spleen cells and immunogen.

4) Cytotoxic cells generated with syngeneic chemically modified sarcoma cells react with unmodified syngeneic and allogeneic tumor cells (i.e. there is no apparent genetic restriction in response). Sensitivity of detection of activity against syngeneic tumors has been increased by use of an adherent target cell assay which results in readily apparent cytotoxicity in a 4 hour assay. Cytotoxicity is not restricted to tumor targets insofar as cultured fibroblasts are also susceptible. Cytotoxicity is selective for target cells; freshly explanted lymphoid cells are not susceptible. Evidence against cytotoxicity being mediated by a fetal calf serum component is that cultured syngeneic leukemia target cells (EL4) are not reactive whereas sarcoma tumor lines carried at least five passages in mouse serum (with washes in between) and tested in mouse serum retain reactivity. Also important is that chemically pretreated syngeneic fibroblasts do not stimulate generation of anti-tumor cytotoxic cells in vitro.

5) Cells from both primary MCA-induced sarcomas and tissue culture lines thereof have been adapted to analysis by rapid flow microfluorimetry. Cells react with fluoresceinated anti MuLV antisera even though many of the tumors are not producing intact virions detected by GP70/P30 ratios or culture for infectious MuLV. Specificity of the staining is most consistent with an MuLV related antigen(s) on the surface of tumor cells. Staining is not due to either nonspecific "stickiness" of antibody to tumor cells or surface acquired factors such as medium components or enzymes. Staining can be blocked by preadsorption of anti RLV with purified RLV. Deviation of tumor tissue culture cells from their primary tumor forebearers is apparent from the relatively intense anti MuLV staining of the former. Differences are apparent with the first 24 hours of tissue culture.

Significance to Biomedical Research and the Program of the Institute: Tumor related immunosuppression has been widely reported from both clinical and animal model studies. Our current work shows that lymphoid cells from mice bearing primary MCA-induced sarcomas are capable of generating nearly normal cytotoxic responses in vitro. This finding is in agreement with our similar investigation of patients with malignant melanoma (see Project No. Z01 CB 05016-08 I). Consequently, improvements in the in vitro anti-tumor immune responses of interest to us will be found less in removal of possible suppressor cells from responder cells than in antigen presentation and delivery of functional cytotoxic cells to the tumor targets.

Further development of an in vitro model for generating primary cytotoxic cell responses against syngeneic tumors will offer more opportunities for identifying and manipulating critical factors in the anti-tumor response and moreover will lead to means for generating anti-tumor cells in quantity for in situ immunoprophylaxis and therapy. Of particular relevance is our use of freshly explanted chemically induced tumor cells and early passage lines free of known acquired pathogens which could be immunosuppressive and/or immunogenic.

Finally, successful adaptation of flow microfluorimetry to single viable cell preparations from fresh tumor explants and to cell lines offers multiple opportunities for rapid sensitive analysis of cell surfaces of both infiltrating host cells and tumor cells themselves.

Proposed Course of Project: Next year's major efforts will be directed at the following goals using MCA-induced murine sarcomas and syngeneic hosts or responder lymphoid cells: 1) The nature and specificity of anti-tumor cytotoxic cells generated in vitro. 2) Whether similar anti-tumor cytotoxic cells can be generated and/or function in vivo. 3) Whether these tumors bear unique MuLV related antigens. In addition we will continue two limited studies with human cells: 1) Information from the mouse model system will be applied to generation of cellular cytotoxicity in vitro against autologous human ovarian cell carcinoma and 2) characterization of human cells which mediate antibody dependent cellular cytotoxicity will be pursued.

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PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Lymphocyte Cell Surface Antigens, Normal and Neoplastic

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Mann	Senior Investigator	I NCI
OTHER:	B. Knudsen	Visiting Fellow	I NCI
	C. Murray	Guest Worker	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biologic, functional and chemical characterization of human lymphoid cell surface antigens are under investigation. Methods for rapidly isolating subpopulations of lymphocytes (B cells, monocytes, K cells, T cells) have been developed. Monocytes share B cell alloantigens as do skin macrophages. Multiple genes control the expression of B cell alloantigens and are linked to genes controlling HLA antigens. Studies of two autoimmune diseases, Sicca syndrome and SLE have demonstrated a complex of B cell alloantigens occuring in greater frequency compared to the normal populations, suggesting an autoimmune response gene. The B cell alloantigens of man and mice appear to have arisen from a common ancestral gene by two criteria: 1) antisera detecting murine Ia antigens also react with human B cell antigens precipitating components with similar molecular weights; 2) amino acid sequence of the 34,000 molecular weight chain bearing human B cell alloantigenic determinants is nearly identical sequence with that reported for the murine Ia antigen. Chemical analysis of the HLA alloantigens have demonstrated 5 major peptides. Different HLA alloantigen molecules have demonstrated near complete amino acid sequence homology save for a variable region surrounding a cystine peptide.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of these studies are as follows: a) to isolate and purify human cell surface antigens for molecular and biochemical characterization; b) to develop serologic reagents to detect B cell antigens; c) to relate the presence of cell surface antigens to disease states; d) to assess the biologic function of different cell surface structures in relationship to the immune function of the lymphocytes.

Methods Employed: ^{51}Chromium release and dye exclusion cytotoxicity tests are performed for the evaluation of the presence of cell surface antigens both on normal lymphocytes, and on subpopulations of these lymphocytes. Cell sources used in these studies were from normal donors, donors with various disease conditions and long-term lymphoid tissue culture cell lines. The method for isolation of subpopulations of cells is as follows: monocytes are isolated by adherence to glass surfaces and removed with an EDTA reagent. The nonadherent cells are then plated on plastic surfaces previously coated with antiserum to the Fab component of Ig. This technique isolates the immunoglobulin bearing lymphocytes or B cells. These cells are removed by competition with human Ig. The nonadherent cells from this incubation are then plated on plastic surfaces coated with antigen-antibody complexes. This technique allows the isolation of Fc bearing lymphocytes. These cells are removed by competition reagents. The remaining cells are functional T cells. These subpopulations have been tested for a variety of cell surface antigens. The technique of separation of cells is being employed to investigate the function of different subpopulations of human peripheral blood lymphocytes in in vitro test systems. Subpopulations of cells are isolated by the method described above with the 4 populations resulting. The various subpopulations are used in assays to differentiate the functional properties of subpopulations of cells, in stimulation of allogeneic cells in mixed lymphocyte reaction MLC. One or another or various combinations of the cell populations are incubated with allogeneic cells and standard MLC reactions. The stimulating cells are treated with mitomycin to inhibit DNA synthesis. After a suitable incubation period, the cultured cells are pulsed with tritiated thymidine and assessment of tritiated thymidine incorporation is determined.

In an attempt to determine the presence of B cell alloantigens on skin macrophages, the following techniques were employed: monocytes and B cells from peripheral blood were tested in cytotoxicity assays for the presence of B cell alloantigens. Langerhan cells or skin macrophages were isolated from epidermal biopsy material. Positive and negative sera determined by cytotoxicity tests of peripheral monocytes were then tested by fluorescence and cytotoxicity against the Langerhan cells. The fluorescent technique employed the use of staff A protein conjugated with fluorescene. Absorption studies with either of the two cell types was also performed in order to determine sharing of a particular human Ia (B cell) alloantigen in these two cell types.

Cytotoxicity assays on isolated B cells were performed in family studies where known genetic crossovers had been described. These studies were carried out in order to determine if more than one gene was controlling the expression of the human B cell alloantigen.

In addition to employing reagents detecting human B cell alloantigens, antisera known to detect murine allotypic Ia antigens were employed in family studies.

Studies of the molecular characteristics of human B cell alloantigens were performed by labelling cell membrane components with tritiated amino acids. The cell membrane components were solubilized by nonionic detergents and purified by gel filtration chromatography and lectin column purification employing the lectin concanavalin A. After elution of the adhered antigen with alphamethylmannose, appropriate antisera (or control normal sera) were incubated with the isolated material and the antigen-antibody complexes precipitated with the Cowan strain Staph. aureus organism. The antigen-antibody complexes were dissociated with sodium dodecyl sulfate in the presence of a reducing substance mercaptoethanol. These dissociated materials were electrophoresed on polyacrylamide gels containing sodium dodecyl sulfate. Gel slices were counted in a liquid scintillation counter and the migration of the isolated labelled proteins assessed.

Large quantities of lymphoid cells are being employed to determine the structural characteristics of the HLA alloantigens. HLA alloantigens are solubilized from cell membrane preparations with non-ionic detergents or with the enzyme papain. These solubilized materials are purified by gel filtration chromatography, ion exchange chromatography, and immunoabsorbant columns employing the use of antisera directed against β_2 microglobulin. The isolated HLA alloantigens are then cleaved at the cystine or methionine residues or by acid cleavage. These peptides are isolated by DEAE chromatography and amino acid sequence analysis performed on the isolated material. The quantities of lymphoid cells needed to pursue the chemical and structural characterization of HLA alloantigens are being provided by NCI contract NO1-CB-12078. The isolation and structural analysis is being done in collaboration with Dr. Jack Strominger, Harvard Biological Laboratories, Cambridge, Massachusetts.

Major Findings: A large number of antisera have been developed which detect individual B cell alloantigen specificities. These antisera have been rendered specific for B cell alloantigens by adsorption with pooled platelets. Serologic studies have revealed these B cell alloantigens to be in detectable quantities on the surfaces of B lymphocytes and monocytes, both peripheral blood and on Langerhan cells (skin macrophages). B cell alloantigens appear to be nondetectable on K cells, the Fc bearing subpopulation of human peripheral blood lymphocytes and on T cells. Other findings subsequently suggest that these antigens are on T cells by assay procedures more sensitive than cytotoxicity. Genetic studies employing these antisera have demonstrated that more than one gene appears to control the expression of the B cell alloantigens (this has been a controversial issue in that a number of investigators believe that the B cell alloantigens are controlled by a single gene with multiple alleles). Studies in 4 families where HLA cross-overs have been determined have demonstrated at least 3 genes controlling the expression of the human B cell alloantigens in man.

Studies in patients with the disease Sicca syndrome have demonstrated the reaction of five antisera to be increased in frequency in this disease population compared to the frequency of appearance of the reaction of these sera in the normal population. Family studies suggest the necessity for the presence of two of these alloantigens in a cis-trans position in those individuals having the particular disease. However, it appears that there are other contributing factors in that siblings not demonstrating the disease at the time tested, may also possess these antigenic determinants in this cis-trans position. Studies have also been performed in another autoimmune disease, systemic lupus erythematosis. One particular antiserum appears to detect antigens on the cells on a majority of these patients compared to a relatively low frequency in the general population. Antisera showing increased frequency of reactions with cells derived from patients with Sicca syndrome also appear to be incresed in reactivity frequency in patients with systemic lupus erythematosis. The observation that a series of alloantigens are present in these two autoimmune disease states suggests the presence of a human immune response gene or genes which may be related to the autoimmune manifestation of disease, at least in the two populations studied. The presence of B cell alloantigens have also been determined in the Pima Indian population. This population of individuals is interesting because of the high incidence of diabetes mellitus (approximately 45%). The distribution of HLA alloantigens has been found to be somewhat limited, however, a reasonable diversity has been demonstrated. In contrast one particular B cell alloantigen specificity has appeared in all the Pima Indians thus far studied. The relationship of appearance of this antigen to diabetes in this population remains to be determined. These studies were supported in part by an NCI contract N01-CB53890 to Duke University.

Alloantisera detecting murine Ia antigens have been found to react with human peripheral blood B cells in family and population studies. The reactivity patterns of these sera suggest the detection of alloantigens segregating within the family in association with a particular HLA haplotype. Absorption studies have confirmed this observation. In contrast, human alloantisera detecting B cell alloantigens do not appear to react with murine B cells. Immunoprecipitation studies with the murine Ia antisera employing human cells demonstrates a molecular component similar to or identical with that precipitated with human alloantisera detecting B cell alloantigens.

Studies have been performed in attempts to assess the biologic significance of cells bearing the human B cell alloantigenic determinants. In mixed lymphocyte cultures it appears that the presence of a cell bearing the B cell alloantigens is necessary in either the stimulating or responding cell population in order for the mixed lymphocyte reaction to occur. T cells stimulated with T cells do not undergo transformation or tritiated thymidine corporation. If macrophages or B cells are added either to the stimulating population or to the responding population, an MLC reaction will occur. T cells and macrophages will respond to an isolated T cell population in allogeneic situations. Alloantisera reacting with B lymphocytes from the stimulating population have been used in blocking experiments in an attempt to demonstrate the particular antigenic determinant responsible for stimulation. These antisera have been shown to block the mixed lymphocyte reaction suggesting the presence of the human Ia-like antigens on peripheral blood T cells or a small subpopulation of this isolated cell population. It therefore appears that cells possessing these human Ia-like antigens are necessary in order for allogeneic recognition.

Quantities of the HLA gene products and HLA-B gene products as well as the human B cell alloantigens are being isolated from lymphoid tissue culture cells. The specificity and purity of the isolated antigenic material is tested by cytotoxic inhibition. The chemical characterization of these isolated components is being assessed in studies performed in collaboration with Dr. Jack Strominger of the Harvard Biologic Laboratories in Cambridge, Massachusetts. Five major peptides have been isolated from the HLA-A gene product and four from the HLA-B gene product. The difference in the number of peptides isolated is dependent on an acid hydrolizable residue in the HLA-A gene product. A large degree of homology has been demonstrated in the amino acid sequence analysis of these major peptides save for the appearance of a variable sequence of amino acids near the most N terminal cystine component. Studies of other isolated HLA-A and HLA-B gene products is necessary in order to determine if this variable region is responsible for the immunodominant allogeneic determinant. Sequence studies on the 34,000 dalton component from isolated B cell product demonstrates a large degree of homology with the reported amino acid sequence of 34,000 dalton component of the murine Ia antigen. This homology together with the demonstration of the serologic reactions of antisera detecting murine allotypic determinants suggests a common ancestral gene for the B cell alloantigen products.

Significance to Biomedical Research and the Program of the Institute: Human B lymphocyte alloantigens appear to be analogous to the B cell alloantigenic system in mice. Genes controlling these antigens in mice appear to be linked to or associated with the ability of the animal to respond to particular antigens. Identification of the human alloantigens together with the description of the fine structure of the genetic complex determining these alloantigens may show the association of immune response genes to antigens in man. HLA alloantigens have been found to be associated with increased frequency in certain disease states. This association in no instance is complete. All individuals bearing a particular HLA alloantigen do not demonstrate a particular disease and conversely in not all instances of the occurance of a certain disease is there the expression of a specific HLA alloantigen. This observation suggests that the presence of closely linked gene(s) plays some role in the pathogenesis of the diseases. Our observations in the two autoimmune disease states, Sojren's syndrome and SLE have extended the hypothesis that certain genes or genes closely associated with the expression of particular B cell alloantigen(s) play a role in the disease state. Rather extensive studies in gluten sensitive enteropathy have demonstrated a common alloantigen in these individuals. This alloantigen appears to be a receptor for the wheat germ protein gluten which is the precipitating factor in this disease. Identification of these gene products and determination of their functional significance in the immune response is of fundamental biologic importance. Structural analysis of HLA alloantigens approaches several biologic questions. The protein structure of cell membrane components of lymphocytes is unknown. The HLA antigenic markers provide a resource for the isolation and purification of specific cell membrane structures. The structural analysis of cell membrane components will provide information as to differences that determine their individual antigenicity. Cell membrane structural changes as well as antigenic changes are thought to occur in neoplasia. Therefore, the structural studies on HLA gene products may provide a prototype for studies of antigenic changes that occur in neoplastic transformation.

Proposed Course of Project: The studies will be continued in the determination of B lymphocyte alloantigens both in the description of the number of genes coding for the presence of these antigens as well as the determination of the appearance of these antigens in several disease states. A large study has been initiated with the determination of HLA alloantigens and B cell antigens in a diabetic population allergic to insulin. This study provides a unique opportunity to determine if a specific immune response gene is responsible for the allergic state in these patients. Small amino acid sequence differences are present in the beef and pork insulin. The identification of a specific B cell alloantigen in individuals allergic to a particular kind of insulin will provide evidence for a specific immune response gene in man.

Studies will continue in attempts to demonstrate the biologic function of the human Ia antigens. Preliminary studies suggest that cells bearing these alloantigens are human suppressors, both in mixed lymphocyte reaction and antibody synthesis. Studies are also underway to determine the significance of the appearance of particular HLA alloantigens in the autoimmune disease states Sojren's syndrome and systemic lupus erythematosis. Amino acid sequence studies will be continued in the isolated and purified HLA-A and HLA-B gene products. In addition, structural analysis will be continued on the various gene products that are represented in the expression of B cell alloantigens. Attempts will be made to correlate the molecular structure with the property of antigenicity.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05011-10 I

PERIOD COVERED
October 1, 1977 through June 30, 1978

TITLE OF PROJECT (80 characters or less)
Rhesus Monkey Histocompatibility Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. R. Neefe, Jr. Investigator I NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Immunology Branch

SECTION
Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rhesus monkey alloantisera raised by planned immunization were evaluated for lymphocytotoxic antibodies by (1) titration against peripheral blood lymphocytes of selected monkeys; (2) typing of random unrelated monkeys and of monkey families by standard microcytotoxicity methods; (3) absorption analysis. Such methods have resulted during the past year in an increase of recognizable RhLA serologically defined antigens from 21 to 24 including 12 in the A series and 12 in the B series.

DR typing with primed lymphocytes has been compared with DR typing by homozygous typing cells. Both appear to detect the product(s) of a locus or loci linked to RhLA in family studies, but disparities between the two methods in random unrelated monkeys suggest there may be at least two loci. Seven antigens expressed predominantly on B lymphocytes have been studied. Four are represented in the monkey families and these behave genetically as alleles of a single RhLA-linked locus.

Project Description

Objectives: To establish and maintain a colony of pedigreed rhesus monkey families for study of the genetics of transplantation and as an eventual source of donor-recipient pairs for transplant experiments.

This work is being conducted at Litton-Bionetics under contract NO1-CB-64106. Dr. Bruce Maurer is the Principal Investigator. Dr. Neefe is the Project Officer.

Methods Employed: Sera are raised by immunization with skin graft and peripheral blood lymphocytes of selected monkeys previously typed for RhLA major histocompatibility complex antigens and appropriately matched to related or unrelated donors. Sera are analyzed for serologically defined antigens by titration against known positive and negative monkeys, by microcytotoxicity typing of families and large random unrelated populations, and by standard statistical analysis of patterns of reactivity observed. Selected sera are further analyzed for monospecificity by absorption and titration.

Monkeys are typed for MLC locus (DR) antigens by performing mixed lymphocyte cultures. Lymphocytes are primed by previous exposure to respond in secondary culture only to a particular antigen; proliferative response to a test lymphocyte in culture indicates the test lymphocyte bears the antigen.

Monkeys are typed for antigens expressed predominantly on B lymphocytes after a procedure designed to enrich a population of peripheral blood lymphocytes for true immunoglobulin-bearing cells. Extensively washed lymphocytes are allowed to adhere to anti-Fab attached to plastic and, after washing, the B cells are competed off with excess free immunoglobulin. These cells are typed by the standard complement-dependent microcytotoxicity procedure. Breeding is designed to produce RhLA homozygotes, RhLA identical siblings, and to enlarge existing families of full siblings.

Major Findings: Analysis of new alloantisera has resulted in improved definition or new identification of 24 RhLA alloantigens, 12 in the A series and 12 in the B series. This represents an increase of three in the past year. All except 3 are defined by more than one serum. In most cases, sera defining an antigen have very similar frequencies and are very highly correlated in 2 by 2 Chi-square analysis in a random population of monkeys. About 80% of the gene products of the two loci can be identified.

Within families, primed lymphocytes appear to detect the same antigen as homozygous typing cells described in the previous report; moreover, the antigen segregates with the RhLA haplotype. This indicates the predominant factor in producing proliferative responses in the monkey, as in other species, is linked to the major histocompatibility complex. However, in unrelated monkeys homozygous typing cells and primed lymphocytes frequently give disparate responses. This may indicate the relevant locus is complex with two or more components. This possibility is being explored.

Fifty-two random unrelated monkeys have been typed with a panel of 57 sera previously identified as having possible preferential reactivity with B lymphocytes. Seven groups of highly correlated sera have been found and are thought to identify Ia or DS antigens. Four of these are represented in the families; they appear to segregate with RhLA and to be the products of a single locus. Breeding has resulted in three live births, with several pregnancies still in progress.

Significance to Biomedical Research and the Program of the Institute: Transplantation may be important to cancer therapy in several ways. For example, bone marrow transplantation may be used to "rescue" patients from otherwise fatal hemopoietic toxicity of intensive chemotherapy. Or, specific immunotherapy involving transplantation of lymphoid cells sensitized to tumor antigens may be useful in some cancers. In developing such techniques it may be useful to use as an experimental model an animal species, such as the monkey, which is closely related to man. Successful exploitation of such a model requires detailed knowledge of the major histocompatibility complex of the species.

Proposed Course of Project: Major emphasis will be upon identification of MLC loci and upon development of antisera for these determinants. Efforts to further improve definition of the RhLA serologically defined antigens by raising and analyzing new sera and to expand the quantity and immunogenetic usefulness of the family members will continue. The Principal Investigator is terminating June 30, 1978; future work will be carried on at his new place of employment outside of NIH.

Publications

Maurer, B. A., Jones, J. A. and Neefe, J. R.: Definition of 17 rehesus monkey histocompatibility antigens including one new antigen. Tissue Antigens 11:1-19, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05016-09 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

In Vitro Tests of Immunity in Patients with Melanoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
OTHER:	W. D. Terry	Associate Director for Immunology	I NCI
	R. J. Hodes	Head, Immunotherapy Section	I NCI
	S. A. Rosenberg	Chief, Surgery Branch	SURG NCI
	R. I. Fisher	Senior Investigator	M NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients participating in an NCI immunotherapy protocol are being tested for effects of a melanoma vaccine, BCG alone, and chemotherapy on cytotoxic humoral and cellular immunity against melanoma vaccine cells and other relevant cell culture lines. Results of in vitro testing have demonstrated that: 1) greater than 90% of melanoma patients receiving the melanoma vaccine generated cellular and/or humoral immune responses against one or more of the vaccine cells; 2) PBL from melanoma patients except for those receiving chemotherapy had a normal capacity for generating cytotoxic cellular responses in vitro, and to this extent were not immunosuppressed; and 3) treatment of melanoma patients with a nonspecific immunostimulant (BCG) did not increase the average in vitro natural killer or K cell (ADCC effector) cytotoxic immune activities.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project have been to carry out in vitro tests for cytotoxic humoral and celluar immunity to determine 1) if patients respond immunologically to the melanoma vaccine and 2) if immunologic responsiveness correlates with disease progression. Patients being tested are associated with an NCI melanoma immunotherapy protocol.

Methods Employed:

1) Direct, Cell-Mediated Cytotoxicity: Cytotoxicity is measured by the release of ^{51}Cr from labelled target cells incubated overnight with immune cells. Target cells are prepared from vaccine melanoma cell lines maintained in tissue culture. Normal and immune lymphoid-attacking cells are prepared from human peripheral blood. Peripheral blood cells are purified by centrifugation with Ficoll-Hypaque. Studies of multiply transfused humans have shown that cytotoxicity in this assay is specific for direct cell-mediated reactions and independent of conventional complement, humoral antibody, and nonspecific toxins.

2) Lymphocyte Dependent Antibody (LDA) Mediated Cytotoxicity. This cytotoxic pathway is also measured by a chromium release assay. After ^{51}Cr labelling, melanoma target cells are washed by centrifugation and then incubated for 1/2 hour with heat-inactivated serum or plasma containing LDA. Target cells are again washed and then incubated with nonsensitized attacking lymphoid cells from normal donors for 4 hours.

3) K Cell Activity. This test is basically carried out as an LDA mediated cytotoxicity assay. Known LDA-containing, poly specific antisera from multi-transfused donors are used as sources of LDA, and peripheral blood lymphoid cells from melanoma patients are used as effector cells. By titering the dose of effector cells, K cell activity (i.e., which will elicit LDA mediated cytotoxicity) from patients° cells can be compared with the counterpart from normal donors° cells.

4. Complement-Dependent, Antibody-Mediated Cytotoxicity. The complement assay is a two-stage, ^{51}Cr-microcytotoxicity test utilizing rabbit complement and vaccine melanoma cell lines.

5. Natural Killer Cell Activity. Cytotoxicity is measured against an allogeneic ^{51}Cr labelled target cell, K562, known to be a sensitive indicator for natural killer cell activity. The assay runs 6 hours and utilizes Ficoll-Hypaque purified peripheral blood lymphocytes.

6. Cytotoxic Cellular Memory. Ficoll-Hypaque purified peripheral blood lymphoid cells are mixed with x-irradiated or mitomycin C treated allogeneic cells in medium with AB positive human serum. After 3 days co-incubation in vitro, cells are harvested, adjusted to a known viable cell concentration, and tested for cytotoxic activity against ^{51}Cr-labelled target cells in an over-

night direct cell-mediated cytotoxicity assay (see above). As in all of the cell-mediated assays, panels of target cells are used to assess specificity of the cytotoxic reactions.

Major Findings: As of December, 1977, 120 melanoma patients have been tested for humoral and cellular immune activity. Results have demonstrated that 18 of 20 patients who received the melanoma vaccine and were frequently tested during the first two months on the protocol, developed significant cellular immune cytotoxicity against at least one of the vaccine cell lines. None of 7 patients receiving BCG alone, who were similarly tested, generated such activity. None of the approximately 100 patients on the protocol who did not receive the melanoma vaccine and were tested at less frequent intervals, developed cellular cytotoxicity against any of the vaccine cell lines. Cytotoxic cellular memory, as evidenced by in vitro antigen-stimulated development of cytotoxic activity against vaccine cell lines, has also been evaluated. Half of approximately 20 patients receiving vaccine generated memory cell activity at some stage of treatment. Such memory activity has been negative in each of 50 patients not receiving vaccine. Tests for the ability of peripheral blood leukocytes to generate primary cellular immune responses in vitro have demonstrated that the average response of cells from melanoma patients, approximately 30 tested, is equivalent to the average response of cells from normal donors except for the patients receiving chemotherapy, approximately 12, whose average response is suppressed. Optimal recovery of PBL from tumor patients required strict adherence to the original Boyum procedure, deviations from which led to fallacious indications of immunosuppression. Cytotoxic activity has been measured against panels of target cells following "primary" (non-vaccinated donors) or "secondary" (vaccinated melanoma patients) in vitro immune responses. Thirty melanoma patients have been tested, 10 of whom have received the vaccine. Patterns of reactivity indicate that specific immune responses are generated combined with an underlying level of natural killer cell-like activity. Tests for natural killer and K-cell cytotoxic activity have demonstrated that neither therapy with vaccine (15 patients tested) nor therapy with BCG alone (11 patients tested) has increased either of these types of activities relative to pre-treatment values or normal donor controls.

Significance to Biomedical Research and the Program of the Institute: In vitro cytotoxicity tests are being used to assess the effectiveness of immunotherapy for melanoma patients. These tests help determine 1) if patients are immunosuppressed; 2) if the level of immunosuppression correlates with disease progression; 3) if a vaccine preparation is defective as indicated by failure to stimulate response in any patient; and 4) if the vaccine has provided non-specific immunostimulation.

Proposed Course of Project: Patients on the melanoma immunotherapy protocol will continue to be tested for humoral and cellular cytotoxic immune responses. Because next year will be the terminal year of this project, and support will be at a reduced level, the major effort will be directed at solidifying important findings which are currently preliminary because of limited data.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05017-08 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biological Significance of HLA Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. N. Rogentine, Jr., M.D.	Senior Investigator	I NCI
OTHER:	D. P. van Kammen, M.D.	Senior Investigator	AP NIMH
	E. S. Gershon, M.D.	Senior Investigator	AP NIMH

COOPERATING UNITS (if any) S. Leiken, Children's National Medical Center, Washington, DC R. Fontana, Mayo Lung Project, Mayo Clinic, Rochester, MN, E. Hersh, M.D. Anderson Hospital, University of Texas, Houston, TX, J. Docherty, West Haven VA Hospital, West Haven, CT

LAB/BRANCH

Immunology Branch

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NCI, NIH, Bethesda, Maryland 20014

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

HLA antigens have been associated with a variety of diseases. Our studies concentrate on malignant diseases, disorders of immunity and psychoses. We are testing the hypothesis generated by our previous work that long-term survival in leukemia and lung cancer is due to the possession of certain HLA antigens. Other diseases studied include melanoma, acute schizophrenia and manic depressive psychoses. It is too early to report on HLA and survival in leukemia and lung cancer, although at diagnosis there appears to be an abnormal distribution of HLA-A antigens (see text) in leukemia. HLA-A2 is elevated significantly ($p<.02$) in acute schizophrenia; there appears to be no HLA association with manic-depressive psychoses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Definition of the natural biological significance of certain human cell surface antigens is our objective. We have been studying the well-known HLA antigen system. It has importance for organ transplantation, but our emphasis is upon the relationship of HLA antigens to the natural function of the human organism.

We are seeking the significance of HLA antigens by investigating their frequency disturbances in different diseases. If such disturbances are found, and many have been, we can design further studies on the role of the antigen in the pathophysiology of disease.

Methods Employed: HLA antigens are assayed by dye exclusion cytotoxicity tests. These are being conducted on contract by Drs. F. Ward and A. H. Johnson of Duke University (N01-CB-52800).

Major Findings: Studies of the association of HLA antigens and disease continued in several areas. Most of the studies are in progress at present.

On the basis of previous work in acute leukemia and lung cancer, we hypothesized that the possession of certain HLA antigens conferred survival advantage to patients with these antigens. Testing this hypothesis requires the prospective typing of a large number of cancer patients. Through a collaboration with the Children's Cancer Study Group (previously Acute Leukemia Group A), we have completed typing all their newly diagnosed acute leukemia cases. With 696 patients typed, this is the largest series ever studied. We are asking two questions: a) are HLA antigen frequencies abnormal at diagnosis (implying a susceptibility/resistance factor to the development of the disease), and b) does survival correlate with possession of certain HLA antigens. Analysis of data from these 696 patients indicated a significant, $p<.005$, perturbation of HLA-A locus antigen frequencies with HLA-A2 being elevated. No data is yet available on survival.

A similar study of Stage I lung cancer patients was completed with the NCI Lung Cancer Project at the Mayo Clinic. Over 200 patients have been typed. Data analysis has not been done yet.

We are continuing to type melanoma patients at NCI and the M.D. Anderson Hospital and to assay postimmunization (with a killed allogeneic melanoma cell vaccine) sera for anti-HLA activity. This data has not been completely analyzed yet.

We are continuing to assay HLA antigens in patients with well-documented acute schizophrenia, since there is some evidence for disordered immunity in this disease. In our studies there continues to be a preponderance, significant at $p<.02$, of HLA-A2. More patients are being tested in collaboration with Dr. John Docherty of the West Haven V.A. Hospital.

We assayed HLA antigens in patients and family members with manic-depressive psychoses, since it is known that forms of this disease are genetic. The data fails to suggest a linkage with HLA.

Finally, this contract serves to obtain the occasional HLA typing needed in the course of work of various investigators at NIH. This amounts to 10-15% of all HLA typing done.

Significance to Biomedical Research and the Program of the Institute: Cell surface molecules are involved in important recognition phenomena controlling normal growth and development. Malignancies arise when this recognition mechanism fails. Thus the study of these molecules is of critical importance to understanding normal growth and development and malignancy. Fortunately many cell membrane structures are antigenic, allowing identification by standard immunologic techniques. It is our purpose to study selected plasma membrane antigens and their respective antibodies.

The cell surface histocompatibility antigens are a product of an extremely polymorphic genetic system which must have important survival value for the individual irrespective of its role in allograft rejection. For example, the possession of given HLA specificity may confer, by mechanisms unknown, susceptibility (or resistance) to a particular disease. Therefore, one rational approach to assessing the natural biological significance of HLA antigen is the study of the association of disease with these antigens. If such associations are found, they have double significance. Closer medical observations of individuals possessing disease-associated HLA antigens may result in earlier diagnosis and better control of the disease. In addition, such knowledge may help us design experiments to probe the disease's basic pathophysiology.

Proposed Course of Project: A variety of patients with diseases, especially malignancies and disorders of immunity, will be typed for HLA antigens. In particular, we will continue to collect data in melanoma. Correlations with survival will be studied in the leukemia and lung cancer patients already collected; this will take several years of follow-up time. We will continue typing schizophrenics because of the suggested increase in HLA-A2 and the hypothesis that this may be an autoimmune disease.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05018-08 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Killer Lymphocyte Damage to Target Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. A. Henkart	Senior Investigator	I NCI
OTHER:	C. K. Neels	Microbiologist	I NCI
	C. L. Stephens	IPA Investigator	I NCI
	C. B. Simone	Clinical Associate	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project previously established a model system to study the effects of killer lymphocytes on foreign cell membranes by using antibody coated planar lipid bilayers as target membranes for attack by normal human lymphocytes. To extend this project, Dr. Stephens has been measuring the electrical properties of target cell membranes using microelectrodes for intracellular recording. Complement mediated killing of these cells is accomplished by a dramatic and early loss of membrane potential and electrical resistance. Under some conditions cells can recover from this membrane damage and escape subsequent death. To obtain more detailed information on the nature of this membrane lesion, Dr. Simone has performed experiments using resealed red blood cell ghost membranes with fluorescent dextran molecules of varying defined sizes inside. These experiments show that complement lesions allow 40,000 molecular weight molecules to escape but not those of 80,000 mw, while antibody dependent lymphocyte mediated killing of these targets allows molecules of 500,000 but not 2,000,000 molecular weight to escape. Thus we can define a discrete sized pore in the membrane after damage to the target cell, confirming the concept that killer lymphocytes operate by this mechanism.

Project Description

Objectives: Our overall objective is to define the mechanisms by which lymphocytes destroy foreign cells. To this end we have dissected the killing process into discrete steps which can be studied independently. In this project we have concentrated on the membrane lesion induced by lymphocytes in the target cell. Based on previous work by us and other, we hypothesize that killer lymphocytes implant "ionophoric" molecules into the target cell membrane, making the membranes leaky to ions and leading to colloid osmotic lysis. This project seeks experimental support of this hypothesis by examining target membrane damage by electrical measurements and by chemical studies of permeability.

Methods: Two principal approaches have been taken towards these objectives. In the first we have used standard methods of electrophysiology for intracellular electrical recording from large target cells (primary nerve and muscle cultured cells, neuroblastoma cells). To date most of our results have come with complement mediated lysis of these cells. In these experiments the cells are TNP modified and coated with anti-TNP, and then electrical recording begun followed by addition of complement. The other approach has involved making simple membranes as targets for antibody dependent cell mediated cytotoxicity (ADCC). We have performed an extensive series of experiments with liposomes containing DNP-cap-PE as targets, using both small unilamellar liposomes and large multilamellar vesicles, using a variety of geometries for killing, and using various lipid compositions. We have also used TNP modified erythrocyte ghosts and smaller vesicles derived from such ghosts as targets. In all these studies with simple membranes, the fluorescent dye carboxyfluorescein has been the principle marker followed for release into the medium. In the experiments with resealed ghosts as targets, derivatives of dextran containing covalently bound rhodamine have been used in addition to carboxyfluorescein. The release of these compounds was followed using fluorometry and fluorescence microscopy.

Major Findings: Electrical recording from target cells being attacked by antibody plus complement show that a very early consequence of complement action on membrane is a complete loss in membrane potential and electrical resistance. This follows the addition of active complement within 1-2 minutes but is not seen with heat inactivated complement, or in the absence of antibody or hapten on the cell. In these cells complement induced microscopic changes in the cells are not seen until 15-20 minutes, and trypan blue staining follows still later. Interestingly, the membrane potential and resistance decreases are not sufficient to insure the subsequent death of the cell, as shown by the following experiments. If the medium is changed within two minutes after the electrical changes are observed, the cells will recover their original electrical potential and resistance and not show trypan blue uptake. Also, if more dilute complement is used, which lyses only 50% of the target cells, all the target cells recorded from show the electrical changes; half subsequently recover from the membrane damage. Currently we are perfecting techniques to extend these studies to T cell killing and ADCC of these target cells. In our other approach using simple target membranes we have been unable to obtain lymphocyte mediated release of carboxyfluorescein greater than controls using antibody coated liposome targets under the conditions described above. On the other hand resealed red cell ghosts are excellent targets in an ADCC reaction using carboxyfluorescein. However smaller "right-side out" vesicles are not detectably lysed by a similar ADCC reaction, suggesting that the size of target cell can be important and providing a possible explanation for our failure with liposomes. Using red cell ghost targets a discrete sized pore induced in the membrane by killer lymphocytes could be detected by using rhodamine-dextrans of known molecular weight. Thus while complement treated ghosts will allow passage of 40,000 mw dextran but not 80,000 mw dextran, lymphocyte mediated killing produced much larger holes which allowed passage of 500,000 but not 2,000,000 molecular weight dextran.

Significance to Biomedical Research and the Program of the Institute: The process of lymphocyte destruction of foreign cells may be one of the most important mechanisms for the immunological rejection of allografts and tumors in vivo. Studies such as ours of the details of this process can be carried out in vitro and studied at the cellular and molecular level. Such studies are needed to complete our basis knowledge of the details of this important immunological defense process. Our studies have clearly shown that the target cell membrane is probably the primary target of killer lymphocyte attack, and the present studies have enabled us to clarify the nature of the membrane lesion by rigorous methods. Our recent results reinforce the concept that cell mediated killing acts by implanting a discrete pore in the target membrane and thus is parallel to the mechanism of complement mediated killing.

Proposed Course of Project: Electrical recordings from cells undergoing immune attack will be increasingly devoted to cell mediated killing mechanisms--especially killer T cells, but also ADCC killing. The red cell ghost target project is being extended by using other internal markers besides dextran (proteins) and by using other effector cells besides lymphocytes (e.g. monocytes and neutrophils) in ADCC. However, we and others have been unable to lyse red cells with killer T lymphocytes. We hope to use nucleated cell targets with these fluorescent markers inside and study C mediated as well as T cell killing in parallel studies to what we have done.

Other approaches to T cell killing will be taken. Tumor cell plasma membrane vesicles filled with carboxyfluorescein will be prepared, and their binding to T cells studied using fluorescence microscopy and the fluorescence activated cell sorter. The possible release of dye by a killing mechanism will be sought. If these studies are promising, transplantation antigens will be solubilized and partially purified and then reconstituted into carboxyfluorescein filled liposomes. Experiments parallel to those with plasma membrane vesicles will then be carried out.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05021-07 I

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Antigens Determined by the Major Histocompatibility Locus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Head, Transplantation Biology Section	I NCI
OTHER:	T. H. Hansen	Postdoctoral Fellow	I NCI
	N. Shinohara	Visiting Associate	I NCI
	J. K. Lunney	Postdoctoral Fellow	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Immunology Branch

SECTION Transplantation Biology Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 3.5	PROFESSIONAL: 2.5	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are being directed towards an understanding of the role of the products of the major histocompatibility complex in the cellular interactions of the immune response. Projects can be divided into three major categories: 1) Characterization of Major Histocompatibility Antigens: Congenic resistant strains of mice have been reciprocally immunized to produce antisera detecting the products of limited regions of the MHC. Antigens determined by genes in the Ir region termed Ia antigens have been detected and are being studied functionally and structurally; 2) Characterization of the Response to Major Histocompatibility Antigens: The effect of genetic background on the ability to respond to particular Ia and H-2 antigens is being assessed; and 3) Characterization of the Receptor Site for Histocompatibility Antigens: Anti-idiotypic antibodies are being produced and their effects on in vitro and in vivo parameters of graft rejection are being assessed.

Project Description

Objectives: 1) To produce antibodies against the products of defined regions of the major histocompatibility complex. 2) To characterize the reactions of these antibodies with the cell surface of lymphocytes, and to determine the nature of the cells bearing individual antigens. 3) To attempt to produce anti-idiotypic antibodies against the receptors on these antibodies which detect cell surface histocompatibility antigens.

Methods Employed: Congenic resistant strains of mice differing only at their major histocompatibility loci have been reciprocally immunized in order to produce antibodies of known, restricted specificity. The antibodies have been characterized by assays of complement-mediated lymphocytotoxicity and by fluorescence microscopy using a fluoresceinated rabbit antimouse immunoglobulin as a developing agent.

The presence of unexpected specificities in these sera reacting with subpopulations of lymphoid cells has led to the development of methods of distinguishing subpopulation reactions. Additivity studies for cytotoxicity reactions and fluorescent staining have been performed with these reagents in combination with reagents of known specificity for B or T subpopulations of lymphoid cells. Also, reactions with fractionated lymphoid cell populations have been performed. T-cell derived tumors have been used for the absorption of the congenic antisera in order to reveal further specificities for antigens expressed preferentially on B cells. In addition, use has been made of congenic resistant animals bearing recombinant H-2 alleles in order to determine the genetic localization of many of the new antigens.

Antibodies against individual antigenic specificities have been isolated by adsorption onto lymphoid cells bearing those specificities, and the antibody-coated cells have then been injected back into syngeneic animals in order to immunize against the "receptor" portions of the antibody molecules. Sera have been collected from these animals after multiple immunizations and are being characterized for the presence of antibodies capable of reacting with the appropriate antihistocompatibility antigen receptors of antibodies and cells. Tests include inhibition of cytotoxicity, binding to solid-phase immunoabsorbents, passive hemagglutination, and in vitro and in vivo blocking of the immune response to tissue antigens.

Similar protocols are also being applied to antibodies raised in inbred rats differing at multiple histocompatibility loci, and to cross-species systems.

Major Findings: 1) Several of the antisera produced in congenic resistant mice have been shown to contain specificities additional to those expected in these strain combinations. These specificities have been shown to be determined by genes mapping within the Ir region of the murine major histocompatibility locus, and to be expressed preferentially on B cells. Similar antigenic specificities have simultaneously been described by other laboratories, and the name Ia (Ir associated) antigens has been adopted to describe this new class of cell-surface antigens. Anti-Ia antibodies have been demonstrated in several H-2 antisera by absorption with T cell tumors.

2) The discovery of one of these Ia specificities on the B cells of a congenic recombinant mouse differing from another mouse only in the Ir region has been found to correlate with the previously described "one-way" mixed lymphocyte culture (MLC) reaction in this strain combination. This has led to the hypothesis that these Ia antigens may be associated with or responsible for the MLC reaction. This hypothesis has been confirmed experimentally using genetically defined MLC combinations and separated cells. Antibodies to distinct Ia antigens have been shown to specifically inhibit the MLC reaction at the level of apparent block of the stimulator cell.

3) Anti-Ia antibodies have been found to specifically block the Fc receptor of B lymphocytes. In addition, one other genetic locus not linked to H-2 has been found to determine antigen(s) associated with the Fc receptor.

4) Anti-idiotypic reactions against the anti-H-2 antibodies have not yet been demonstrable.

5) An antibody response to a previously unrecognized H-2 antigen has been found and has been determined to be genetically controlled by a non-H-2-linked locus. The antibody response to H-2.32 has been found to be under similar control.

6) Antibodies to Ia like antigens of the rat have been detected in anti-AgB antisera by absorption of the antisera with rat platelets. By polyacrylamide gel electrophoresis antigens precipitated by these antisera have been shown to be of the same size as mouse Ia antigens.

7) Cytotoxicity tests and absorption analyses on a congenic anti-Ia antiserum have suggested the presence of another subregion within the Ir region of the H-2 complex. This subregion has been given the name I-E.

8) Analysis of antihistocompatibility antisera by gel electrophoresis of labelled cell surface antigens has demonstrated a new H-2 product determined by a previously unrecognized H-2 locus within the D region, termed H-2L.

9) A mutant mouse, the BALB/c-H-2^{db} has been studied and has been found to lack all specificities associated with the L molecule.

Significance to Biomedical Research and the Program of the Institute: 1) The Ia antigens appear to provide a new cell-surface marker for the B subpopulation of lymphocytes. In addition, the localization of the genes determining these antigens to the Ir region may indicate that these B cell surface structures are involved in the T cell-B cell collaboration necessary for many immune responses. If so, these antigens may provide markers for dissecting the mechanism of that collaboration.

2) The specificity of the receptor for histocompatibility antigens should reside in the variable portion of the heavy and light chains of the relevant antibody molecules. Thus, anti-idiotypic antibodies against such receptors might be expected to distinguish those cells capable of reacting against individual histocompatibility antigens. Such antibodies, if obtained, could thus provide a new approach to modification of the immune response to cell surface antigens.

3) Understanding of the genetic control of response to H-2 antigens may make possible the modification of these responses in order to produce tolerance.

Proposed Course of Project: 1) Other antisera raised between congenic resistant strains of mice differing by known regions of the major histocompatibility complex will be screened for additional Ia specificities. The cellular distribution of these specificities will be determined (i.e., T cells vs B cells), and lymphoid tumors of known cellular origin will be used to absorb differential activities.

2) Models for the functional properties of these antigens which may explain their genetic localization to the Ir region will be further explored. These will include MLC reactivities, cellular cooperation models and cellular cytotoxicity models.

3) The relationship between Ia antigens and the Fc receptor will be pursued. Other non-H-2 antigens also associated with the Fc receptor will be investigated, as well as the Fc receptor of other cell populations such as macrophages and T cells.

4) Attempts at immunization against the receptors of anti-H-2 antibodies and cellular receptors for alloantigens will be continued. Xenogenic immunization will be attempted. An attempt will be made to raise monoclonal anti-H-2 and anti-Ia antibodies by cell fusion techniques.

5) Extensive testing as outlined above will be required in order to determine whether or not anti-idiotypic antibodies have been produced in any of our immunization protocols. If we can demonstrate the presence of such antibodies serologically, we will then attempt to purify them by selective immunoabsorption on columns bearing the appropriate immunoglobulins. The effects of these antibodies on in vivo cellular and humoral responses to tissue grafts will then be tested.

6) Additional strain distribution studies will be performed in order to determine whether the new locus of H-2 (H-2L) has other alleles which can be detected in other haplotypes.

Publications

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Shinohara, N., Ricks, J., Hansen, T. H., and Sachs, D. H.: Genetic control of immune response to the $H\text{-}2D^k$ private specificity, H-2.32. I. Low responsiveness of the B10 congenic mice to the H-2.32 determinant. J. Immunol. 119: 1732-1737, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05023-07 I

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Transplantation Antigens of Swine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	S. A. Rosenberg	Chief, Surgery Branch	S NCI
	R. L. Kirkman	Clinical Associate	I NCI
	J. K. Lunney	Postdoctoral Fellow	I NCI
	M. W. Flye	Investigator, Surgery Branch	S NCI

COOPERATING UNITS (if any)

NIH Animal Center, Poolesville, Maryland

LAB/BRANCH Immunology Branch

SECTION Transplantation Biology Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.4	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A breeding program has been carried out starting with two miniature pigs from different sources and selecting offspring according to tissue typing procedures aimed at defining the major histocompatibility complex of this species. By this procedure three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. MLC reactions, serologic typing, and skin graft survival times all indicate that these animals are homozygous for a major histocompatibility locus analogous to HL-A of man or H-2 of the mouse.

Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for tissue typing and transplantation; 2) Purification and characterization of the major histocompatibility antigens of this species; and 3) Assessment of the immunologic parameters involved in tolerance to liver allografts in this species. Inbreeding in brother-sister fashion within each of these herds will be continued with the ultimate goal of establishing truly inbred animals if possible.

Project Description

Objectives: To develop and maintain three strains of miniature swine, each homozygous for a different set of histocompatibility antigens at the major histocompatibility locus (MSLA). The animals are used for in vivo experiments in organ and tissue transplantation and as a source of large numbers of cells from which cell surface antigens are isolated, purified, and characterized chemically.

Methods Employed: Unlike classical inbreeding schemes which require about twenty generations to produce homozygosity, the approach being used with these swine involves selective breeding on the basis of histocompatibility typing of parents and offspring.

Four males and four females were purchased from commercial sources, and mating pairs were selected to be as varied as possible in order to assure the selection of different histocompatibility genes. Skin grafts were exchanged between the members of each pair and sera were obtained from the animals two weeks after the rejection of the grafts. The sera were tested for cytotoxic antibodies by lymphocytotoxic typing, and pairs which produced strong cytotoxic antibodies were bred.

Offspring from each breeding were tested serologically to determine which histocompatibility antigens had been inherited from the parents, and offspring which could be shown to possess the same antigen combinations were selected for further breeding. This process has now been repeated for six consecutive generations.

Transplantation of tissues in these animals has been performed in the large animal facility of the Surgery Branch in Building 14. Methods have been developed for the transplantation of split thickness skin grafts from the ear to the dorsal thorax. Allografts are always placed side by side with autografts as a control. The use of ear skin permits very accurate assessment of viability of the grafts and determination of rejection times. Surgical techniques for vascularized grafts (kidney and liver) are being developed.

Chemical purification studies on swine transplantation antigens are being performed using detergent solubilized cell surface preparations from spleen and lymph node cells. Initial studies involved radiolabelling of the surface antigens with tritiated leucine by published methods, and the assessment of molecular weight of those components which react with alloantisera by the use of SDS polyacrylamide gels. Methods are currently being developed for handling larger quantities of histocompatibility antigens without the use of radiolabelled amino acids.

Major Findings: 1) Naturally occurring anti-red cell antibodies of swine are cytotoxic to lymphocytes (unlike their human counterparts) and must be absorbed from sera with packed red cells before leukocyte typing is performed.

2) Skin grafts between appropriate third and fourth generation animals were rejected in 8-14 days, and the rejection was accompanied by the production of high-titered lymphocytotoxic antibodies. Boosts with lymphocytes have been carried out in several related combinations and have yielded large quantities of high-titered antiserum.

3) Breeding of further generations has continued to be successful.

4) Typing of the fourth generation offspring yielded three breeding pairs of animals homozygous for serologically determined transplantation antigens. We therefore now have the capacity to breed three different herds of swine, each homozygous for a different MSLA antigen.

5) MLC assays of parents and offspring showed a close correlation between this parameter of histocompatibility and the serologically determined transplantation antigens. Since this type of correlation has previously been observed in studies of man (HLA), monkey (RhLA), mouse (H-2), rat (AgB) and dog (DLA), this finding indicates that we are selectively breeding for homozygosity at a major histocompatibility complex (MHC) homologous to that of other mammalian species.

6) Skin graft survival has been found to be prolonged within each of the homozygous herds. The mean survival time for grafts within homogyzous herds was 11.8±.89 days, while the mean survival time of skin grafts between animals of the three different herds was 7.0±.36 days.

7) Renal allografts within the DD homogyzous herd appeared to survive indefinitely despite minor histocompatibility antigen differences. Rejection occurred following allografts within the other two herds, with variable kidney survival times.

8) Skin grafts to DD recipients maintaining a DD allografted kidney showed marked prolongation of survival.

9) DD kidneys transplanted after skin graft rejection by DD recipients were rejected in hyperacute fashion, but no antibody was detectable.

10) Alloantisera between the three herds have been analyzed by gel electrophoresis using detergent solubilized cell surface antigen preparations. Peaks were obtained at 45,000 molecular weight corresponding to the mouse H-2 antigen analog. Peaks at 35,000 and 28,000 molecular weights corresponding to mouse Ia antigens were also observed. Both by size criteria and by genetic criteria these antigens thus appear to be the precise homologs of H-2 and Ia antigens of the mouse.

Significance to Biomedical Research and the Program of the Institute: One of the major problems in the study of cell surface antigens of human beings is lack of control of genetic constitution. The use of mice and rats as experimental models avoids this problem, but creates two new ones: 1) physiologically and anatomically these animals are often so different from human beings as to make comparisons and applications of findings difficult. 2) The size of these rodents makes it extremely difficult to obtain sufficient cells and tissues to permit quantitative chemical characterization of relevant cell surface antigens.

For both of these reasons it is desirable to have animals of size comparable to human beings, whose genetic constitution with respect to histocompatibility can be controlled. The miniature pig, which attains an adult weight of about 200 lbs, is ideal for this purpose, and to date the experimental breeding pattern outlined above appears to be working well.

Proposed Course of Project: The breeding plan and typing will be continued. Lines will be established and separated, so that herds of any required size in each line can be produced.

Experiments in collaboration with the Surgery Branch will be continued in order to: 1) characterize the immune response to transplantation of skin, kidney, and liver in pigs across defined histocompatibility differences, and 2) to determine the basis of liver allograft tolerance which has previously been reported in pigs.

Studies of the MSLA antigens at the biochemical level will be continued. These will center first on isolation and purification, and then on chemical and immunologic characterization.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05033-07 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. I. Fisher	Senior Investigator	M	NCI

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A controlled, randomized trial comparing immunotherapy to chemotherapy in stage I and stage II malignant melanoma has been initiated. A total of 137 patients have entered the trial, but it is too early to evaluate the results.

Project Description

Objectives: This project is designed to study various approaches to the immunotherapy of human malignancies.

Methods Employed: A new protocol (designated IB-2) was initiated in August 1975. This protocol will evaluate the effect of two types of immunotherapy or one type of chemotherapy on the remission duration and survival of patients with stage I (level 4 or 5) and stage II malignant melanoma. Patients are stratified by stage, site of primary, and for stage II patients, presence or absence of clinically palpable lymph nodes and the number of histologically positive lymph nodes. Following stratification, they are randomized to receive no further therapy (this is standard treatment for these patients) or chemotherapy with methyl-CCNU or immunotherapy with BCG, or immunotherapy with BCG plus a vaccine that consists of three tissue culture-grown allogeneic melanoma cell lines. These cell lines were a gift from Dr. Donald Morton, UCLA, and are grown in PPLO-free conditions at Litton-Bionetics under the supervision of Dr. Edwin Matthews. Cells are treated with the enzyme neuraminidase to remove sialic acid (and thus render them more immunogenic) and frozen until ready for use.

All patients are worked up by the Immunology Branch or the Surgery Branch. Following randomization, they are assigned to the Medicine Branch for chemotherapy, the Immunology Branch for immunotherapy, and the Surgery Branch for follow up if there is no further treatment.

Bloods are drawn during pre-randomization work-up and subsequent to randomization assays of cell-mediated and humoral immunity are performed.

Major Findings: As of April 1, 1978, 126 patients have been randomized into this protocol. The state of the trial is summarized in the following table:

	Control	MeCCNU	BCG	BCG & Vaccine
Total Patients Entered	29	31	31	35
Recurrences	13	10	17	14
Deaths	9	7	11	8

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see CB-05016-I). In addition PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy studies will explore the clinical effectiveness of manipulating the immune system in patients with cancer, and will also provide new information about the biology of the tumor-host relationship.

Proposed Course: The IB-2 protocol will be continued.

Publications

Terry, W.D. Present status and future directions for cancer immunotherapy. *Gann Monogr. Cancer Res.* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05034-06 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Characterization and Isolation of Human Lymphocyte Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. B. Dickler	Senior Investigator	I NCI
OTHER:	R. M. Blaese	Head, Cellular Immunology Section	M NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.25	PROFESSIONAL: 0.5	OTHER: 0.75

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to characterize and isolate human lymphocyte subpopulations and evaluate the function of these populations in the immune response of both normal and diseased individuals. Recent work under this project has shown that anti-HL-A alloantisera contain antibodies against antigens which are found primarily on B lymphocytes and which are associated with B lymphocyte Fc receptors. In addition it was found that the Fc receptors of B lymphocytes and UL cells differ both in their avidity for Ig complexes and in the fact that the Fc receptors of UL cells do not appear to be associated with "Ia-like" antigens. Human peripheral blood mononuclear cells cultured in vitro with the antigen KLH-TNP generated significant numbers (compared to controls without antigen) of plaque-forming cells measured with SRBC coated with staph protein A in the presence of rabbit anti-human Ig, but not when measured with SRBC or SRBC-TNP.

Project Description

Objectives: To characterize and isolate human lymphocyte subpopulations and evaluate the function of such populations in the immune response in both normal and diseased individuals.

Methods Employed: Lymphocytes from normal human donors were reacted with alloantisera and then evaluated for the presence of bound antibody and function of Fc receptors by fluorescent techniques. Alloantisera were fractionated by absorption with and elution from platelets. Antigen-antibody complexes and double marker fluorescence techniques were used to evaluate the avidity of lymphocyte subpopulations for Ig complexes. Human peripheral blood mononuclear cells were cultured in vitro with or without soluble protein antigens in order to generate antibody secreting (plaque-forming) cells measured in a plaque-forming cell assay using a variety of indicator cells. Subpopulations of lymphocytes were purified or depleted using a variety of techniques including gradient centrifugation, rosetting, anti-Ig columns, G-10 Sephadex columns, nylon wool columns and adherence. Various markers were employed to evaluate purity of populations including fluorescent detection of surface Ig and Fc receptors, E-rosette formation with SRBC and latex particle ingestion.

Major Findings: Certain anti-HL-A alloantisera contained antibodies which inhibited the binding of complexed Ig to B lymphocyte Fc receptors. After absorption with platelets the alloantisera lost HL-A reactivity but retained the ability to react with B lymphocytes and inhibit Fc receptor function. Conversely, the HL-A antibodies eluted from platelets reacted with all lymphocytes but did not inhibit binding to Fc receptors. The Fc portion of the inhibiting antibodies was not required since $F(ab')_2$ fragments also produced inhibition. These data suggest that human B lymphocytes bear "Ia (immune response gene associated) like" antigens and that these antigens are associated with B lymphocyte Fc receptors. B lymphocytes and UL cells were shown to have Fc receptors which were distinct. UL cells' Fc receptors had a much higher avidity for Ig complexes and were not associated with "Ia-like" antigens. These differences may be of functional importance.

Human peripheral blood mononuclear cells from several normal individuals have been cultured in vitro with or without antigen (KLH-TNP). Those cultures with antigen generated signficant numbers (compared to controls without antigen) of antibody secreting cells as measured in a plaque-forming cell assay. Plaque forming cells were observed using SRBC coated with staph Protein A in the presence of rabbit anti-human Ig but not with SRBC or SRBC-TNP. A culture period of 6 days, antigen concentration of 10-50 μg/ml, and a cell concentration of 2.5×10^6/ml were found to be optimal.

Significance to Biomedical Research and the Program of the Institute: Characterization and isolation of lymphocyte subpopulations is of fundamental importance for an understanding of the nature of the immune response.

By analogy to the murine system, identification of human alloantigens associated with Fc receptors and primarily expressed on B cells may be important for understanding immunoregulation and interactions between immunocompetent cells, and may help characterize the genes responsible for such regulation.

Proposed Course of Project: (1) The antigen-driven plaque forming cell response will be used to evaluate the conditions (including various proportions of B and T lymphocytes and accessory cells) needed to obtain antigen-specific plaque-forming cell responses to soluble protein antigens *in vitro* using human peripheral blood cells. (2) If *in vitro* antibody responses to soluble antigens can be obtained, this system will be used to evaluate a) Ir gene function in man; b) the role of various cell populations in the antibody response in man; and c) the role of Ir genes in human disease.

Publications

Arbeit, R., Henkart, P., and Dickler, H. B.: Differences between the Fc receptors of two lymphocyte subpopulations of human peripheral blood. *Scand. J. Immunol.* 6:873-878, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05035-06 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Characterization of the Lymphocyte Receptor for Complexed Immunoglobulin.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. B. Dickler	Senior Investigator	I NCI
OTHER:	R. J. Klingenstein	Clinical Associate	I NCI
	D. H. Sachs	Head, Transplantation Biology Section	I NCI
	D. L. Nelson	Investigator	M NCI

COOPERATING UNITS (if any)

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LAB/BRANCH
Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.375	1.125	0.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to characterize lymphocyte Fc receptors genetically and functionally. Recent findings indicate that the Fc receptors of B cells and T cells but not UL cells nor macrophages are associated with Ia antigens. In addition, a subpopulation of UL cells, the effector cells in antibody-dependent cell-mediated cytotoxicity was shown not to bear Ia antigens. In the case of the B cell, the Fc receptors and Ia antigens appear to be distinct molecules, and the association appears to be ligand induced. There is also a single polymorphic non-H-2 locus which determines at least 4 distinct antigens associated with the Fc receptor. In one haplotype, this locus has been shown to be linked to the M locus. Studies with pharmacologic agents suggest that B lymphocyte Fc receptors are linked to the lymphocyte cytoskeleton. These findings indicate that Fc receptors may play a key role in immunoregulation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To evaluate the genotypic and phenotypic expression of the Fc receptor; 2) to evaluate the role of this receptor in the immune response.

Methods Employed: Heat-aggregated immunoglobulin and/or antigen-antibody complexes are fluorochrome or radioactive isotope labelled, and then allowed to interact with isolated lymphocytes or macrophages from animals. Binding is assayed by U.V. and phase microscopy, the Fluorescence Activated Cell Sorter (FACS), or by isotope counting. The interaction is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both lymphocytes and complexes. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Functionally, the effect of Ig complexes on the humoral immune response (plaque-forming cell assays) and antibody dependent cell-mediated cytotoxicity (ADCC) are analyzed.

Major Findings: The Fc receptors of murine B lymphocytes are associated with Ia antigens in that anti-Ia antibodies specifically inhibit binding of Ig complexes to Fc receptors. However, the two moieties appear to be distinct molecules in that capping most Fc receptors does not appear to alter the distribution of Ia antigens. In addition, quantitative analysis of Ia antigens using the FACS before and after binding of complexes to Fc receptors and/or capping Fc receptors did not alter the amount of Ia antigens detected. Thus, since the two moieties appear distinct, and since inhibition of detection of Ia antigens was not detected upon binding of complexes to Fc receptors, the association does not appear to be steric in nature. The most plausible explanation for the inhibition of detection of Fc receptors by anti-Ia antibodies is that the binding of a ligand to Ia induces an association with Fc receptors.

Antigens determined by at least one polymorphic non-H-2 locus are also associated with B cell Fc receptors. The antigens determined by this locus appear to be mainly expressed on B lymphocytes and appear to be distinct from Fc receptors on the basis of capping experiments. The locus itself is not linked to the major histocompatibility complex, the Ig allotype locus and is not sex-linked. It is, however, closely linked to the M locus.

A portion of T lymphocytes also have Fc receptors but these appear to be of either lower affinity or are present in fewer numbers per cell than the Fc receptors of B lymphocytes. They also appear to be associated with Ia antigens on certain T lymphocytes.

The Fc receptors of macrophages and UL cells do not appear to be associated with Ia antigens. In addition, most UL cells including those that mediate ADCC lack Ia antigens.

Capping of B cell Fc receptors by Ig complexes was moderately inhibited by cytochalasin B and markedly inhibited by a combination of cytochalasin B and colchicine suggesting that Fc receptors of B lymphocytes may be linked to the lymphocyte cytoskeleton.

Significance to Biomedical Research and the Program of the Institute: The receptor for complexed antibody has been shown to play a key role in antibody-dependent cell-mediated cytotoxicity. This immunologic mechanism may plan an important part in host response to tumors. The Fc receptor/Ia antigen association is hypothesized to be important in regulation of the humoral immune response and collaborations between immunocompetent cells.

Proposed Course of Project: 1) Studies of the Ia-Fc relationship by capping of Ia antigens and other cell surface markers followed by evaluation of Fc receptors. 2) Use of pharmocologic agents and techniques evaluating mobility of cell surface proteins to evaluate the relationship of Fc receptors to other surface molecules and to cytoskeletal structures. 3) Analysis of the requirements for alteration or aggregation of Ig which allow binding to Fc receptors. 4) Analysis of the role of Fc receptors in regulation of the immune response using various functional assays.

Publications

Dickler, H. B., Arbeit, R. D., Cowing, C. O., Henkart, P. A., Kubicek, M. T., Nelson, D. L. and Sachs, D. H.: Studies of the relationship between Ia antigens and Fc receptors on various cell populations. In Miescher, P. A. (Ed.): Immunopathology VIIth International Symposium. Basel, Schwabe & Co. Publishers, 1977, pp. 66-73.

Dickler, H., Kubicek, M., Arbeit, R. and Sharrow, S. O.: Studies on the nature of the relationship between Ia antigens and Fc receptors on murine B lymphocytes. J. Immunol. 119: 348-354, 1977.

Nelson, D. L., Sachs, D. H. and Dickler, H. B.: Antibody-dependent cellular cytotoxicity effector cells lack Ia antigens. J. Immunol. 119: 1034-1040, 1977.

Dickler, H. B., Ahmed, A. and Sachs, D. H.: B lymphocyte Fc receptor associated non-H-2 antigens are determined by a single polymorphic locus which is linked to the Mls locus. J. Exp. Med. 146: 1678-1692, 1977.

Dickler, H. B., Kubicek, M. T., Arbeit, R. D. and Sharrow, S. O.: The association between Ia antigens and Fc receptors on murine B lymphocytes is probably ligand-induced. In McDevitt, H. O. (Ed.): Ia Antigens and Ir Genes. New York, Academic Press, Inc., 1978, pp. 185-193.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05036-06 I

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Staphylococcal Nuclease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Head, Transplantation Biology Section	I NCI
OTHER:	D. S. Pisetsky	Investigator	I NCI
	M. D. Geier	Chemist	I NCI
	J. A. Berzofsky	Expert	M NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	2.0	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies directed against idiotypic determinants on anti-staphylococcal nuclease antibodies from different mouse strains have been produced in rats and can be detected by the inhibition of antibody-mediated inactivation of nuclease. These anti-idiotypes have been shown to be specific for the combining site (variable regions) of antinuclease antibodies, since extensive absorption with normal mouse immunoglobulins fails to diminish their activity, and since antibodies to other sites on the Ig molecule do not inhibit inactivation. As nuclease is an antigen, the response to which is under control of H-2-linked immune response genes, it was of interest to determine the relationship between the idiotype and other genetic loci. By screening a variety of strains and offspring from appropriate matings between strains for the presence of idiotypes and other markers, it has been shown that idiotype expression is linked to the heavy chain allotype marker and not to the immune response gene locus. A number of recombinants between different idiotype markers have been found and may provide tools for fine-structure mapping of heavy chain variable region genes.

Project Description

Objectives: The antibody response to staphylococcal nuclease (NASE) is under genetic control by gene(s) located in the Ir region of the mouse major histocompatibility complex (H-2). Since the antibodies produced to certain regions of this antigen have been previously shown to demonstrate restricted heterogeneity, we wish to determine whether or not the relevant Ir gene determines which variable region immunoglobulin products are produced. If the Ir gene is found to exert quantitative but not qualitative control over idiotypic antibody production, then a search for a distinct "T cell receptor" for nuclease (i.e., other than known IgV regions) must be anticipated.

Methods Employed: 1) Antibodies to NASE have been prepared in groups of mice of a variety of strains (both high and low responders) differing in H-2 type or allotype or both. Certain of these have been purified by affinity chromatography. Inbred Lewis rats have been immunized with the purified anti-NASE antibodies obtained from immune ascites from A/J mice, SJL mice, B10.A(2R) mice, and BALB/c mice after immunization with NASE. Immunoabsorptions with normal immunoglobulins from the same strains have been used in order to determine whether or not antibodies reactive with the variable region (i.e., anti-idiotypic antibodies) have been produced.

2) Anti-idiotypic reactions have been quantified by the inhibition of antibody-mediated enzyme inactivation. Such reactivities have been screened against anti-NASE antibody populations from the other strains of mice in order to determine the possible genetic linkage of idiotype to allotype and/or H-2 type.

3) Backcross animals have been screened for antibody levels and for allotype and idiotype expression in order to determine linkage of idiotype to other genetic markers.

4) Antibodies to nuclease have been separated on affinity columns into subpopulations directed against different determinants of nuclease. The reactions of anti-idiotypic antisera with these subpopulations have been characterized in order to determine new idiotypic markers.

5) Antibodies to individual determinants of nuclease have been measured in antisera from high and low responder strains. A radioimmunoassay using labelled polypeptide fragments of nuclease has been developed for this purpose.

Major Findings: By two separate criteria, anti-idiotype antibodies have been obtained. 1) After exhaustive absorption with whole normal A/J ascites or repeated passages over affinity chromatography columns to which normal A/J ascites was bound, antibodies remained which reacted with A/J anti-NASE but not with B10.A anti-NASE antibody. Prior to absorption these rat antibodies contained precipitating antibodies to either type (A/J or C57BL10) of antibody but no precipitating antibodies remained following absorption. 2) The rat anti-A/J anti-NASE antibodies prior to and following absorption reacted with the combining site of anti-NASE antibodies as evidenced by their ability to inhibit the anti-NASE antibody inactivation of NASE in enzymatic assay.

Among (B10.AxA/J)xB10.A backcross animals the A/J anti-NASE idiotype was found to be linked (p§.005) to heavy chain allotype. However, a large recombination frequency was found (7-10 percent). The use of a very sensitive assay for allotype indicated that this recombination frequency was not the result of faulty allotypic typing. Also, progeny testing of the putative recombinant animals showed approximately 50 percent of the offspring to also have recombinant phenotypes, further indicating that these were true recombinant animals. These recombinant haplotypes are being inbred to provide new recombinant strains.

A backcross between (BALB/cxCB-20) and CB-20 was performed and examined for idiotype segregation. The same phenomena of idiotype inheritance and recombination frequencies were observed. These recombinants are also being preserved.

Following multiple boosts with antigen, congenic high and low responder animals (B10.A and B10, respectively) were both found to make comparable levels of antibody. However, the proportion of antibody against the carboxy terminal third of the nuclease molecule was very different between these two strains. This indicates that *H-2*-linked *Ir* gene control must exert an effect at the B cell level as well as at the T cell level in cell interactions of the immune response.

Significance to Biomedical Research and the Program of the Institute: Many of the antigens being studied in this laboratory are difficult to obtain in pure form (such as transplantation antigens and tumor antigens), and it is therefore important to have a well-characterized model protein on which initial experiments can be performed. In choosing models both for mechanistic studies and for studies in manipulation of the immune response, staphylococcal nuclease is a particularly attractive protein. The immune response to this antigen has previously been shown in this laboratory to be under genetic control by an *H-2*-linked *Ir* gene.

The development of anti-idiotypic reagents directed toward antibodies under the control of an *H-2*-linked *Ir* gene provides another handle to study the mechanism of *Ir* gene function. These antibodies may help to determine whether the specificity of T cell recognition of antigens depends on similar variable region gene products to those responsible for B cell or antibody specificity. They may also lead to possible methods of controlling *Ir* gene expression.

Proposed Course of Project: There is suggestive evidence from other workers that the T cell antigen receptor shares the same idiotype as an antibody directed against that antigen. To study such a role for the idiotype in the generation of antinuclease antibodies, the effect of the anti-idiotype on the production of antinuclease antibodies will be investigated. As the stimulation as well as the abolition of antibody production are both possible expressions of anti-idiotype interaction with immune cell receptors, studies will be designed to assess both. An alternate approach, the effect of anti-idiotype antibodies on the generation in vitro of antigen-specific T cell factors, will be explored. If these two approaches fail to provide evidence for the role of the idiotype on T cells, an attempt will be made to immunize with immune T cells rather than antibody in order to determine whether another "idiotype" can be distinguished for the T cell receptor.

The anti-idiotype preparations currently available are heterogeneous with specificities directed against a variety of antinuclease antibodies. To define more precisely the specificities represented, antibodies against subregions of the nuclease molecule will be prepared and the interaction of the anti-idiotype with these fractionated antibodies studied. It is hoped that these fractionated preparations will provide more sensitive reagents in the study of the genetic basis for variable region diversity of antinuclease antibodies, as well as possible study of the detailed structure of the nuclease receptor on T cells.

Each of the recombinant animals which have been found by assessment of idiotype in backcross progeny will be established as a new line. A variety of other variable region markers will then be tested in these animals, in an attempt to develop a linear map of the variable region genes.

Further study of backcross progeny in several different strain combinations will be performed. This should help to determine recombination frequencies more accurately and should also help to provide a better linear map of the variable region. Attempts to determine the basis for sharing of idiotypic determinants between strains will be made by the examination of closely related strains and sublines. This may shed light on the mechanisms for the generation of diversity of antibody specificities.

An attempt to isolate the T cell receptor for nuclease will be made, using nuclease-derivatized nylon. If obtained, presence of idiotype and Ia antigen will be assessed by binding studies.

Publications

Sachs, D. H., Berzofsky, J. A., Fathman, C. G., Pisetsky, D. S., Schechter, A. N., and Schwartz, R. H.: The immune response to staphylococcal nuclease: A probe of cellular and humoral antigen specific receptors. Symp. Quant. Biol. XLI: 295-306, 1977.

Pisetsky, D. S., and Sachs, D. H.: The genetic control of the immune response to staphylococcal nuclease: VI. Recombination between genes determining the A/J antinuclease idiotypes and the heavy chain allotype locus. J. Exp. Med. 146: 1603-1612, 1977.

Berzofsky, J. A., Schwartz, R. H., Schechter, A. N., and Sachs, D. H.: H-2 linked control of the antibody and cellular immune response to nuclease at the level of individual regions of the molecule. In McDevitt, H. W. (Ed.): Ir Genes and Ia Antigens. New York, Academic Press, Inc., 1978, pp. 423-431.

Pisetsky, D. S., Berzofsky, J. A., and Sachs, D. H.: Genetic control of the immune response to staphylococcal nuclease: VII. Role of non-H-2-linked genes in the control of the anti-nuclease antibody response. J. Exp. Med. 147: 396-408, 1978.

Sachs, D. H., Berzofsky, J. A., Pisetsky, D. S., and Schwartz, R. H.: Genetic control of the immune response to staphylococcal nuclease. Springer Seminars in Immunopathol. 1:51-83, 1978.

Pisetsky, D. S., and Sachs, D. S.: Genetic control of the immune response to staphylococcal nuclease. VIII. Mapping of genes for antibodies to different antigenic regions of nuclease. J. Exp. Med. 147: 1517-1525, 1978.

Berzofsky, J. A., Pisetsky, D. S., Schwartz, R. H., Schechter, A. N., and Sachs, D. H.: In Atassi, N. Z. and Stavitsky, A. D. (Eds.): Proceedings of the First International Symposium on the Immunobiology of Proteins and Peptides. New York, Plenum Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05038-06 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to TNP-Modified Syngeneic Lymphocytes in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	A.-M. Schmitt-Verhulst	Expert	I NCI
	C. M. Pettinelli	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

T-cell-mediated lympholysis was generated in vitro against trinitrophenyl-(TNP-) modified syngeneic murine spleen cells. The cytotoxic effectors generated are specific for both the modifying agent and for self major histocompatibility complex (MHC) products mapping in the K or D regions of the H-2 complex. Specificity of the effectors was such that they could not lyse H-2-matched targets in which the TNP group was presented on the targets by insertion of a TNP-fatty acid-dextran conjugate into the cell membrane. However, effectors could be generated by culturing spleen cells with TNP coupled to soluble proteins. Cellular analyses of the mechanism by which TNP-conjugated proteins stimulated cytotoxic responses indicate that: (a) phagocytic accessory cells are required for generating cytotoxicity; (b) interaction of TNP-protein with cell surfaces was not mediated by a phagocytic mechanism; and (c) H-2 restriction is not determined by the phagocytic accessory cells. Changes in the ability to generate TNP-dependent cytotoxicity was induced by injecting mice with trinitrobenzene sulfonic acid (TNBS). The effects were dependent on TNBS dose and time after injection. Short-term priming followed by specific unresponsiveness four months later was observed.

Project Description

Objectives: The objective of this project was to investigate the immunogenetic parameters associated with the in vitro generation of T-cell-mediated immunity of murine spleen cells to syngeneic lymphoid cells modified with the TNP group. More explicitly this project was designed to: (a) further elucidate the fine specificity (i.e., the contribution of the TNP moiety to the specificity) of the cytotoxic effector cells for TNP-modified self reactions; (b) determine whether it is possible to generate cytotoxic effector cells by stimulating with TNP-conjugated soluble proteins, and if so, to determine whether such effectors are H-2 restricted; (c) establish the cellular requirements for generating effectors to TNP-proteins as well as for PFC to TNP; (d) determine whether the same Ir genes controlling cytotoxicity to TNBS-modified cells also influence cytotoxicity generated by TNP-proteins; (e) examine which cell type(s) is defective in the Ir gene controlled response to TNP associated with $H\text{-}2D^d$; and (f) determine whether the cytotoxic and antibody responses to TNP can be modulated by pre-injecting TNBS into mice prior to culture of their spleen cells.

Methods Employed: The methods employed for specific objectives (a) through (f) are indicated below:

The specificity contributed by the TNP group to the cytotoxic effector cell was further investigated by comparing the lytic potential of the effector cells generated by sensitization with syngeneic trinitrobenzene sulfonate- (TNBS-) modified stimulators (which binds to cell surface proteins by covalent linkage) and with cells modified with TNP-stearoyl-dextran (TSD) (which binds to cell surface lipoprotein by noncovalent forces). Specificity was also tested by comparing TNBS- and TSD-modified syngeneic cells for lysis by and cold target cell inhibition of effector cells.

Mouse spleen cells were sensitized with TNP-modified soluble proteins such as bovine gamma globulin (TNP-BGG) and bovine serum albumin (TNP-BSA). The effector cells generated were tested for specificity on TNBS-modified target cells of different H-2 haplotypes, as well as for Ir gene control. Mice were injected with different amounts of TNBS by different routes, and the TNP-specific cytotoxic and antibody-forming potentials were assessed at different times after injection.

Major Findings: The major findings for specific objectives (a) through (f) are summarized below:

(a) Effector cells generated by sensitization with TNBS-modified spleen cells did not lyse nor were they blocked by TSD-modified, H-2-matched target cells which contrasts with TNBS-modified H-2-matched targets. Furthermore, TSD-modified cells were not able to sensitize syngeneic spleen cells, in contrast to TNBS-modified cells.

(b) Effector cells were generated in culture against TNP-BGG, TNP-BSA, TNP-MSA, and TNP-OA. These effector cells were restricted to lyse only TNBS-modified target cells sharing K and/or D region products with the responding cells.

(c) The presence of a glass-adherent, phagocytic, radioresistant, theta-negative cell (accessory cell) was required during sensitization, since no cytotoxicity was observed when both responding and sensitizing populations were depleted of accessory cells. However, cytotoxic responses could be obtained with TNBS-modified or TNP-BSA precultured accessory cell-depleted stimulating cells when unfractionated responding cells were used. The response could be restored in cultures depleted of accessory cells by a purified population of accessory cells syngeneic, semi-syngeneic or allogeneic to the responding and stimulating cells. These results indicated that H-2 restriction is determined by the "modified" stimulating population and not by the accessory cells, and that H-2 homology is not required for cellular interaction(s) involving the accessory cell in this response. Multiple H-2 linked immune response (Ir) genes appear to control the level of cytotoxicity generated against the TNP-modified H-2D restricted response when generated either by TNBS-modified cells or TNP-conjugated proteins.

(d) It was observed that an antibody response to TNP (assayed by PFC) was generated by G-10 non-adherent spleen cells when stimulated with TNP-conjugated proteins, but not when stimulated with TNP-modified cells.

(e) Intraperitoneal injection of mice with TNBS affected the ability of their splenic lymphocytes to generate cytotoxic response to TNP-self but not to alloantigens. With higher doses of TNBS (.02-2.0 mg/mouse), a transient priming effect was observed, followed by an unresponsive state, and finally by the return to normal response potential. At doses below .02 mg/mouse no priming was detected, but unresponsiveness was observed which appeared much later than at higher doses.

Significance to Biomedical Research and the Program of the Institute: The project is of fundamental immunological interest in that it describes a major histocompatibility linked immunological phenomenon involving self recognition. The recognition of self MHC-coded structures in association with foreign antigenic determinants raises the possibility that self recognition is important to autoimmunity and possibly for virally-infected autologous cells. The finding that products of the MHC are important for the antigenic complex recognized as well as for determining immune response potential provides a working hypothesis for bifunctional MHC control of disease susceptibility in those examples of HL-A-associated diseases in man. The demonstration that the same kinds of responses can be generated using soluble antigens raises the possibility that soluble materials become immunogenic by interacting with cell membrane components, which in turn activate cellular immune mechanisms.

Proposed Course of Project: Investigation of this model will continue in order to determine: (a) whether different self MHC products are recognized by proliferating, helper, and cytotoxic T cells; (b) the role of macrophages in these responses and the possible "modification" of macrophage MHC-coded products for recognition of the TNP-modified soluble antigens; (c) whether immune response genes can be identified for specificities other than H-2D^{d}-TNP, and to map such genes; (d) the cell type(s) which express the Ir gene controlling the response to H-2D^{d}-TNP; (e) the mechanisms for priming and suppression of responses to TNBS-injected mice; (f) the mechanisms responsible for the reciprocal relationship between TNP-specific antibody and cytotoxic responses; and (g) whether MHC-restricted cell-mediated responses can be generated against natural antigens such as tissue-specific tumor-specific or viral antigens. (Note: e.g. see new projects on "modification" of self antigens with fetal calf serum and influenza virus.)

Publications

Shearer, G. M. and Schmitt-Verhulst, A.-M.: Major histocompatibility complex restricted cell mediated immunity. Adv. Immunol. 25: 55-91, 1978.

Shearer, G. M., Schmitt-Verhulst, A.-M., Shaw, S., Pettinelli, C., Henkart, P. A. and Rehn, T. G.: The role of MHC genes in T-cell mediated responses to syngeneic modified cells. In Proceedings of Symposium in Molecular and Cellular Biology, in press

Henkart, P. A., Schmitt-Verhulst, A.-M. and Shearer, G. M.: Specificity of cytotoxic effector cells directed against trinitrobenzene sulfonate-modified syngeneic cells: Failure to recognize cell-surface-bound TNP dextran. J. Exp. Med. 146: 1068-1078, 1977.

Schmitt-Verhulst, A.-M., Pettinelli, C. B., Henkart, P. A., Lunney, J. K. and Shearer, G. M.: H-2-restricted cytotoxic effectors generated in vitro by the addition of trinitrophenyl conjugated soluble proteins. J. Exp. Med. 147: 352-368, 1978.

Schmitt-Verhulst, A.-M., Garbarino, C. A. and Shearer, G. M.: H-2 homology requirements for secondary cell-mediated lympholysis and mixed lymphocyte reactions to TNP-modified syngeneic lymphocytes. J. Immunol. 118: 1402-1427, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05043-04 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

AKR Leukemia Lines With T and B Cell Characteristics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. M. Zatz	IPA Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several unusual in vivo murine lymphoma lines were established and studied for surface and functional characteristics. The lines were derived from AKR/J strain mice and are noteworthy in that they exhibit characteristics of both T and B cells. When passaged into young normal AKR/J hosts, the splenic cells appear first like T-cell lymphomas and then acquire aspects of both T and B cells. Characteristics studied were Thy 1 antigen, Ly antigen, Ia antigen, Fc receptor, complement receptor, mitogen responsiveness, and surface immunoglobulin. The Thy^+ tumor cells in lymph node remain Ig negative. It is possible, 1) that tumor cells differentiate to Thy^+ Ig^+ cells in spleen, but not in lymph node or 2) that two tumor subpopulations exist, one of which grows in spleen and lymph nodes (Ig^-), while the other, (Ig^+), grows more slowly and selectively in spleen. Separation of Ig^+ and Ig^- tumor cells by the Fluorescence Activated Cell Sorter (FACS) indicate that Ig^- cells remain Ig^- when passed into secondary hosts. The evidence is still inconclusive as to whether Ig^+ tumor cells undergo a phase of Ig negativity in secondary recipients.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objective of this project is to investigate and characterize several unusual murine leukemias derived from spontaneous tumors arising in thymectomized and/or old AKR mice. This system was selected as a model for a rarely occurring natural variant of AKR leukemia in which the host animal has an atrophic thymus rather than the usual thymoma. Since these tumor cells express both T and B-cell markers, they provide a possible model for study of T and B cell differentiation.

Methods Employed: Passage lines of AKR tumors were established and continued by intravenous injection of 10^5 spleen cells into young AKR hosts. Donor cells were initially derived from tumors arising in old animals, some of which had been totally thymectomized at one month of age. Thus far, 4 lines have been well characterized: AKTB1, 2, 3, and 6. Several additional lines are now being studied.

Cells were studied for various characteristics including Thy antigen, Ly antigen (by adsorption testing, surface immunofluorescence and cytotoxicity), surface immunoglobulin (SIg), in vitro synthesis of immunoglobulin, mitogen responsiveness and antibody-dependent cell-mediated cytotoxicity (ADCC). Fc receptor, complement receptor and Ia antigens were also studied. Kinetic studies on the acquisition of the above markers in vivo were carried out, with preliminary attempts to determine the factors regulating their expression. Ig^+ and Ig^- TB-1 tumor cells were separated by the FACS and passed into secondary recipients. The tumor cells arising in spleens and lymph nodes of these animals were further analyzed by SIg and Fc receptors.

Major Findings:

1. Thy and Ly antigens are present on 90-100% of spleen and lymph node (LN) cells of 3 AKTB tumor lines. Thy was best demonstrated using CBA anti-AKR anti-thy serum from hyperimmunized animals counterstained with fluorescein-labelled rabbit anti-mouse $gamma_2$ immunoglobulin. The anti-Thy sera used were carefully absorbed to remove anti-tumor and anti-B-cell reactivity. AKTB-1 and AKTB-6 are Ly 2.1^+; AKTB-2 and AKTB-3 are Ly 1.2^+.

2. SIg is demonstrable on 80 to 90% of AKTB-1, 2 and 3 spleen cells by the end of each passage. This SIg is of a single class -μ- as demonstrated by specific immunofluorescence and biosynthetic studies, and does not react with anti-alpha chain or anti-gamma chain labelled antibody. Similar results were obtained using polyvalent anti-immunoglobulin.

3. Within each passage of the AKTB-1 tumor line the percentage of SIg-positive spleen cells increases progressively from 15% (around 10 days) to 80-100% (around 24 days) at times when spleens are grossly enlarged (5-10 fold) and cells are Thy-positive. AKTB-6 tumor cells achieve a maximum of 30% Ig positive.

4. Lymph node and thymus cells from AKTB-1 tumor-bearing animals with 90% SIg-positive spleen cells and gross lymphadenopathy are Thy-positive and SIg-negative.

5. Biosynthesis studies demonstrate that the surface immunoglobulin and Ia antigen are endogenously synthesized and not passively acquired. The immunoglobulin of the cell surface is monomeric IgM of the classical B-cell type.

6. Passage of 99.5% pure Ig-FACS separated cells into secondary recipients reveals that these cells remain Ig^- as they grow in the spleens and lymph nodes of these secondary recipients. Because of the greater difficulty in obtaining Ig^+ FACS cells of similar purity (only 92% Ig^+), the data regarding the expression of SIg on tumor cells of secondary hosts receiving FACS separated Ig^+ cells is still inconclusive.

Significance to Biomedical Research and the Program of the Institute: The results of these studies bear upon a number of issues of fundamental importance to both cellular immunology and also to viral leukemogenesis:

1. There are very few cell lines which bear easily detectable T and B cell markers, although a normal subpopulation with such characteristics has been identified in small numbers. This line may represent an abnormal proliferation of this population or an arrested phase of normal lymphoid cell development, implying transitions between T and B cells.

2. The SIg on these cells could represent an abnormal amount of the "normal" T cell immunoglobulin, so that this line could provide a way to examine "IgT" and the putative T cell antigen receptor. The studies indicate, however, that the membrane Ig is of the classical "B" cell type, i.e. monomeric IgM.

3. The difference between spleen and lymph node tumor cells in the expression of SIg within the same animal suggests that this system may be helpful in studying the effect of "microenvironment" on differentiation of tumor cells within the lymphoid system.

4. The ability to isolate SIg from these cells may allow the generation of anti-idiotype antibody in another species, and thus answer the question of whether or not AKR leukemia is a monoclonal disease.

5. Since the AKTB lines bear B cell markers, they may provide a system in which to examine B cell differentiation.

6. Since these doubly marked tumors occur selectively in thymectomized mice or old mice with atrophic thymuses, they may provide information on the target cell for leukemogenesis in a system where the thymic influence is reduced.

Proposed Course of Project: The most intriguing question which these tumors pose is whether or not they may serve as a model for T-, B-, and/or stem cell differentiation. At least two of the TB tumor lines initially appear in spleen as Ig^-, Thy^+, but become Ig^+, Thy^+, while tumor cells in lymph node and thymus remain Ig^-, Thy^+. These findings may be explained by 1) a stable mixture of two tumors which grow at different rates selectively in spleen and lymph node and/or 2) the differentiation of Thy^+ Ig^- cells in spleen to Thy^+, Ig^+ cells, and the block of this step by the lymph node microenvironment. In order to explore these questions further, splenic tumor cells will be fractionated into Ig^+ and Ig^- populations by cell separation techniques, including use of the FACS. These populations will be transferred in vivo and placed in vitro in order to assess the factors which control acquisition of B-cell markers by these subpopulations. These tumor lines have now been adapted to growth in vitro so that their expression of T and B cell markers can be studied as a function of the phase of the cell cycle.

Since the principal investigator on this project has left the NIH, this project has been terminated.

Publications

Schwartz, B. D., Campbell, P. S., Asofsky, R., Greenberg, R. S. and Zatz, M. M.: Synthesis and characterization of membrane immunoglobulin, Ia and H-2 molecules of Thy 1^+ AKR/J lymphomas. J. Immunol. 119: 1706-1710, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05048-04 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Attempted Isolation of Fc Receptor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. B. Dickler	Senior Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolation of the Fc receptor from detergent solubilized radiolabelled murine lymphoid cells was attempted using Ig complexes of various types. No component(s) of the lymphocyte bound specifically to the complexes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Immunochemical characterization of Fc receptors.

Methods Employed: Spleen cells were radiolabelled with ^{3}H-leucine, and the labelled cells were detergent solubilized. The detergent-soluble material was then used as a putative source of labelled Fc receptor.

Binding to immobilized IgG aggregates was found unsatisfactory due to nonspecific absorption problems. Binding to immune complexes formed between TNP-KLH and affinity purified anti-TNP antibodies was tested.

Major Findings: The binding to immobilized IgG aggregates was pursued for some time and attempts were made to reduce the non-specific absorption effects. This technique was eventually abandoned, however, because it could not be rendered specific.

Results using immune precipitates showed binding of material in the molecular weight range of 60-70,000 daltons and 15-20,000 daltons. However, these components were not specific for Ig complexes.

Significance to Biomedical Research and the Program of the Institute: Fc receptors are a potentially important molecule on the lymphocyte surface with regard to immune function. As such it is of great interest to isolate it, characterize it, and raise specific antibodies directed towards it.

Proposed Course of Project: Due to time commitments to other projects, this project has been terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
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PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05049-04 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

F_1 Antiparental Cell-Mediated Lympholysis In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	C. M. Pettinelli	Visiting Fellow	I NCI
	A.-M. Schmitt-Verhulst	Expert	I NCI
	R. B. Levy	Postdoctoral Fellow	I NCI

COOPERATING UNITS (if any)

G. Cudkowicz, Department of Pathology, State University of NY, Buffalo, NY.

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NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse F_1 spleen cells develop cytotoxic activity in vitro against parental cells. The antigenic differences detected appear to be controlled by a structural gene (Hh-1) mapping in or near the D region of the H-2 complex. Although a number of positive correlations have been demonstrated between murine hemopoietic graft rejection in vivo and F_1 anti-parent cytotoxicity in vitro, certain differences have been recently found between these two F_1 anti-parent reactions. These include the observations that marrow graft rejection does not require priming and is not dependent upon thymus-dependent cells, whereas the development of cytotoxic cells requires sensitization and is effected by T-lymphocytes. The same mechanism is also responsible for the rejection of hemopoietic allografts. It has been possible, with the use of radiation chimeras, to demonstrate allogeneic T-cell mediated cytotoxicity against Hh determinants. A number of parallels were observed among NK cell activity, F_1 anti-parent marrow graft rejection in vivo, and F_1 anti-parent cytotoxic potential (as well as that against fetal calf serum components) was enhanced by injecting reponding cell donors with cells enriched for NK activity.

Project Description

Objectives: The objective of this project was to investigate the immunogenetic parameters associated with the generation of murine F_1 anti-parent cytotoxic effector cell in vitro, and to compare them with allogeneic cytotoxicity in vitro, NK cell activity in vitro, and F_1 anti-parent hemopoietic graft rejection in vivo.

Methods Employed: Spleen cells from a number of F_1 mouse strains were sensitized in culture against parental spleen cells. The effector cells generated were assayed on ^{51}Cr-labelled target cells expressing different H-2 haplotypes. NK cell activity was tested in the spleens of mice and a number of parameters were compared with F_1 anti-parent reactivity.

Major Findings: A number of similarities have been observed between F_1 anti-parent marrow graft rejection in vivo and the development of F_1 anti-parent cytotoxic effector cells in vitro. However, a number of differences have been also noted. These include the findings that: a) the in vivo rejection process does not require active immunization, whereas the development of cytotoxic effectors does; b) marrow graft rejection is neither effected by nor dependent upon thymus-derived cells, whereas the F_1 anti-parent effectors and their precursors are T-cells; and c) the recognition responsible for F_1 anti-parent graft rejection appears to involve structures coded for by a gene(s) mapping near H-2D, whereas for F_1 anti-parent cytotoxicity, products of additional genes within the H-2 complex are also important. The comparison of NK cell activity in vitro with F_1 anti-parent marrow graft rejection in vivo and F_1 anti-parent cytotoxicity in vitro indicated that all three phenomena probably share a common cell type which may serve an accessory role in each type of response.

Hemopoietic graft rejection by an Hh mechanism can also be demonstrated between two allogeneic mouse strains. To test cytotoxic reactivity against Hh determinants in allogeneic combinations, irradiated F_1 mice were repopulated with parental stem cells. Splenic parental T-cells from such chimeras were tolerant to the other parental H-2 antigens when expressed on F_1 cells. However, such T-cells were not tolerant to the other parental antigens when tested on homozygous cells (which express Hh determinants). These results suggest that a subpopulation of T-cells recognize Hh determinants in allogeneic cytotoxic reactions.

Since the cytotoxic response to Hh determinants is weaker than that to either alloantigens or TNP-self determinants, attempts were made to enhance the response by injecting F_1 mice with parental and F_1 cells expressing Hh determinants (as a means of priming). Priming effects were obtained with either irradiated parental or F_1 spleen cells, which raised the possibility that NK cells were enhancing the response.

Significance to Biomedical Research and the Program of the Institute: These comparative studies may be of importance for understanding the common and distinct immune pathways for these three phenomena. Such an understanding may be of significance since marrow graft rejection is considered a problem of transplantation, NK cell activity is considered to be an important mechanism for surveillance against lymphoid tumor development, and the cytotoxic response is mediated by a classical T-lymphocyte mechanism.

Proposed Course of Project: The comparative study of these three models will be continued, and various cell types will be studied for their possible contribution to the response. Further mapping studies using recombinant mouse strains will be made in an attempt to map the Hh-1 gene within H-2, as well as to find the additional H-2 region(s) important for F_1 anti-parent cytotoxicity. The possible role of the NK-like cell will be studied with respect to its effects on enhancing Hh-type cytotoxicity and its possible role in other types of cytotoxicity (e.g., modified self and allogeneic). Since it is possible to enhance cytotoxic responses to Hh determinants by pretreating F_1 mice with irradiated F_1 cells, attempts will be made to enhance in vivo marrow and tumor graft rejection potential and to correct the Ir gene defects (non-H-2-linked) for hemopoietic graft rejection by injecting non-responder mice with irradiated cells from high responder (H-2 compatible) mice. The potential of transferred cells in these studies will be correlated with NK activity of the irradiated cell innoculated.

Publications

Shearer, G. M., Cudkowicz, G., Schmitt-Verhulst, A.-M., Rehn, T. G., and Evans, P. D.: F_1 hybrid anti-parental cell-mediated lympholysis: A comparison with bone marrow graft rejection to Hh determinants in vivo and with cell-mediated lympholysis to H-2 alloantigens in vitro. Cold Spring Harbor Symp. Quant. Biol. X: 511-518, 1977.

Kiessling, R., Hochman, P. S., Haller, O., Shearer, G. M., Wigzell, H. and Cudkowicz, G.: Evidence for a similar or common mechanisms for natural killer cell activity and resistance to hemopoietic grafts. Eur. J. Immunol. 7:655-663, 1977.

Cudkowicz, G., Landy, M., and Shearer, G. M. (Eds.) Natural Resistance to Foreign and Leukemia Grafts. Academic Press. New York. In press. publication date).

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U.S. DEPARTMENT OF
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NOTICE OF
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PROJECT NUMBER

Z01 CB 05050-04 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on the Structure and Function of the Constant Portions of Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. M. Segal	Chemist	I NCI
OTHER:	J. F. Jones	IPA Investigator	I NCI
	J. J. Moore	Clinical Associate	I NCI
	E. L. Alexander	Guest Worker	I NCI
	P. H. Plotz	Senior Investigator	A&R NIAMDD
	R. L. Guyer	Staff Fellow	A&R NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to elucidate the mechanisms with which immunoglobulins interact with effectors of the immune system subsequent to complexing with antigens. Models for immune complexes have been prepared by covalently cross-linking antibody molecules using two different bivalent affinity labels and dimethylsuberimidate. Stable oligomers of defined size have been isolated.

Radioactive oligomers have been used to investigate the immunoglobulin binding properties of cells via Fc receptors. Several types of human cells have been isolated; although the number of receptors for IgG varies between cell populations, the affinities of each cell type for oligomeric IgG are the same for each cell within experimental error.

Oligomers have been attached to TNP-modified sheep erythrocytes and the size requirements for eliciting complement-mediated lysis investigated. The results suggest that the ability of IgG to initiate lysis increases gradually with size and that there is no defined unit signal for eliciting lysis. In contrast, cell mediated lysis of oligomer-coated cells is independent of cluster size and depends only upon the number of IgG molecules bound per cell.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To investigate the interactions of the constant parts of immunoglobulins with the various effector systems employed in immune reactions; 2) to study the relationship of antigenic recognition to these interactions and to study the molecular and cellular bases of these phenomena.

Methods Employed: Organic synthesis, gel filtration, ion exchange chromatography, polyacrylamide gel electrophoreses, complement fixation, radiolabelling of proteins, tissue culture, binding assays, antibody-dependent cytotoxicity assays, cell separations.

Major Findings: The binding of affinity cross-linked IgG trimer to several populations of human cells was investigated as an extension of previous studies which were carried out mainly with neoplastic cells or unfractionated murine splenocytes. Cells capable of binding IgG were obtained from human peripheral blood and spleen, but no binding was observed by tonsil cells and thymocytes. All cells tested to date had similar affinities for trimer (i.e., $Ka = 1\text{-}3x10^7\ M^{-1}$) but considerable variation in the number of receptors/cell was observed. Peripheral blood neutrophils had the largest number of receptors ($\sim 3x10^5$ per cell), while circulating monocytes had about one tenth that number. Human peripheral blood lymphocytes typically bound variable (and usually low) amounts of trimeric IgG; however only a small fraction (∿ 10-20%) of the lymphocytes were observed to have receptors for IgG as judged by fluorescent labelling techniques. When the average number of receptors per positive-staining lymphocytes was calculated it appears that human lymphocytes contain a small population of cells with a large ($>10^5$) number of receptors per cell. Preliminary experiments suggest that this population does not contain surface IgM (i.e., are not B lymphocytes). During these studies we observed that Ficoll and Plasmagel isolation procedures cause immunoglobulin (from plasma) to adhere to certain populations of leukocytes, giving rise to artificially low values for the number of receptors/cell. Human cells were observed to bind rabbit, mouse and human IgG with roughly equivalent affinities.

In collaboration with Dr. Paul Plotz a new bivalent affinity label has been developed. The affinity labels are Bis-Dnp dicarboxylic acid esters. These compounds bind specifically to anti-Dnp antibodies via the leaving group, and react covalently with proximal lysine residues. The resulting oligomers have free binding sites and will bind to Tnp-red cells or to Dnp-BSA. Oligomers formed in this way are particularly useful for clearance studies. When injected into rabbits, radiolabelled monomer is cleared from the circulation slowly and at about the same rate as monomeric IgG which had not been treated with the cross-linking reagent. As oligomer size increases the rate of clearance also increases. There is no sudden increase in clearance rates with size, but instead rates increase progressively with the degree of oligomerization.

Oligomers up to pentamer in size formed with Bis-Dnp-pimelic-ester (BDPE), with $(\text{BADL-pro})_2$-EDA (another bivalent affinity label), or with dimethylsuberimidate (a non-specific cross linking agent) do not fix complement to a significant degree. In the presence of multivalent antigens (but not monovalent hapten) those oligomers with free sites became highly anti-complementary, suggesting that in solution, large complexes are required to obtain fixation.

We have also attached radiolabelled oligomers to TNP-sheep erythrocytes and have compared the abilities of oligomers to mediate lysis of the cells in the presence of complement. In general we observed a progressive increase in lytic efficiency with size. That is, a bound immunoglobulin molecule is more efficient at initiating a lytic site when bound as a dimer than when bound monomerically. Similarly, trimer is more effective than dimer and tetramer is better than trimer. IgM behaves similarly to a preparation of IgG oligomers containing mainly tetramer, pentamer and hexamer. No "unit signal" for eliciting the complement cascade could be established, as had been observed previously for dimers of IgE in eliciting degranulation of mast cells.

More recently Tnp-chicken erythrocytes have been coated with oligomers and lysis by Fc receptor-bearing effector cells (ADCC) has been compared with complement mediated lysis. Unlike complement mediated lysis, ADCC appears to be independent of cluster size. Moreover ADCC changes only gradually with the number of IgG molecules bound per target while complement mediated lysis shows a sharp change. These results suggest that the two defense mechanisms might operate at different levels of antibody coating of target cell.

A study of antibody dependent cell-mediated cytolysis in which the antibody is attached to the effector cell (instead of the target) has also been undertaken. It has been observed that several procedures cause radiolabelled antibody to bind to at least two cell types. The most effective method of fixing antibody to cells has been to centrifuge cells through non-aqueous media from a polythyleneglycol solution. Cells treated in this way are, for the most part, viable, and contain more than 10^5 antibody molecules per cell. The antibody remains cell-associated for a least 24 hrs; however we do not yet know how much antibody is associated with Fc receptors, how much is located on the cell surface, or how much is interiorized.

When effector cells containing bound anti-Dnp antibodies are incubated with Tnp coated sheep erythrocytes, they specifically lyse these cells. Moreover, lysis usually goes to completion and cannot be blocked with immune complexes, in contrast to conventional ADCC, where the antibody is bound to the target.

Finally, a second type of Fc receptor has been detected on several populations of cells. This receptor is capable of binding IgM proteins, but not IgG. We have not been able to characterize this receptor to our satisfaction as yet, since it binds monomeric IgM with low affinity. We are hoping to be able to use polymerized IgM to obtain more reliable data.

Significance to Biomedical Research and the Program of the Institute: The recognition of foreign substances by immunoglobulins elicits a number of reactions which normally lead to their elimination from the body. The purpose of this project is to examine the molecular events which occur as a result of antigenic recognition. It is hoped that these studies will enhance our understanding of these processes and allow us to better control immune reactions. An ultimate goal is to enhance the immune response toward neoplastic cells.

Proposed Course of Project: We are continuing our studies of the armed effector cells, and of binding of IgM through specific cell bound receptors. In addition we are planning to examine IgG and IgM binding by several subpopulations of leukocytes in the mouse, including fractionated lymphocytes.

Studies are also underway to assess the relationship between surface density of IgG receptors and the ability of a cell to bind polymerized IgG.

We are hoping to extend the complement studies using liposomes rather than erythrocytes to probe the triggering mechanism in greater detail. Preliminary studies have also been carried out with isolated C1 in order to determine whether antibody clustering increases binding of C1q or activation of C1r and C1s, or both.

Publications

Segal, D. M., Taurog, J. D. and Metzger, H.: Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. Proc. Natl. Acad. Sci. USA 74:2993-2997. 1977.

Segal, D. M. and Titus, J. A.: The subclass specificity for the binding of murine myeloma proteins to macrophage and lymphocyte cell lines and to normal spleen cells. J. Immunol. 120: 1395-1403, 1978.

Alexander, E. L., Titus, J. A. and Segal, D. M.: Quantitation of Fc receptors and surface immunoglobulin is affected by cell isolation procedures using plasmagel and Ficoll-Hypaque. J. Immunol. Methods, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05051-03 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Spontaneously Arising Syngeneic Murine Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	R. B. Levy	Postdoctoral Fellow	I NCI
	T. H. Hansen	Postdoctoral Fellow	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An AKR/J murine carcinoma-like tumor was generated in tissue culture from an undifferentiated fibrosarcoma. An autologous immune response was generated against the carcinoma in vivo and in vitro. Subcutaneous injection of low numbers of the carcinoma cells resulted in a period of tumor growth followed by tumor regression. Lymphocytes from regressing mice were specifically cytotoxic for carcinoma target cells. Cytotoxic effector cells could also be generated in vitro against the carcinoma, and the injection of cells containing effectors protected host mice from a lethal inoculum of the tumor. T-cell-mediated and antibody-mediated cytotoxic studies were performed to determine if new antigens were expressed on the carcinoma cell line. The results from both types of experiments suggested that a loss of a private H-2D^k specificity had occurred with a concomitant gain of a private H-2D^d specificity. Rejection of the tumor in vivo and the generation of cytotoxic effector cells in vitro was shown to be associated with this loss and gain of major histocompatibility complex (MHC) controlled antigens.

Project Description

Objectives: The objectives of this project were to investigate the immunogenetic parameters associated with the rejection by mice from the strain of origin of a cultured tumor cell line. The specific objectives were to:

(a) establish if the observed regression of the tumor cell line by syngeneic mice had an immunological basis, and to determine if cytotoxic effector cells could be generated in vivo and/or in vitro against the tumor;

(b) test whether cells from sensitized cultures would specifically protect syngeneic mice against a lethal inoculum of the tumor;

(c) determine whether the immune response generated against the tumor could be attributed to antigenic changes in the tumor cell line, and if so, to test whether such antigenic changes were associated with products of the MHC and to map these changes within the H-2 complex.

Methods Employed: The methods employed for (a) through (c) were as follows:

(a) Lymphocytes from tumor regressing mice were tested for specific cytotoxic activity and for their potential to generate enhanced cytotoxic responses following secondary stimulation in vitro using a 5-hour ^{51}Cr-release assay on a variety of target cells.

(b) Normal AKR/J mice were injected with lymphocytes containing cytotoxic effector cells as well as with numbers of carcinoma cells which result in death of the mice, and the number of mice surviving was determined.

(c) Spleen cells from normal AKR/J mice were sensitized against a number of stimulating cells differing from the AKR/J responding cells at specific regions or subregions of the H-2 complex. The effector cells generated were tested on a series of target cells differing at specific regions of H-2 using the ^{51}Cr-release assay. Antibody-mediated, complement-dependent lysis of the carcinoma tumor cell line was performed using H-2 region specific antisera.

Major Findings: The major findings are as follows:

(a) During the regression of the carcinoma tumor, syngeneic AKR/J mice were found to have lymphocytes which specifically lysed the carcinoma tumor. Cytotoxic effector cells which lysed the carcinoma could be generated in vivo or in vitro.

(b) AKR/J lymphocytes sensitized in vitro against the carcinoma were capable of protecting AKR/J mice against lethal inoculation of the tumor.

(c) Antigenic changes were detected in the cultured carcinoma cell line by both cellular and antibody-mediated immune reactions. A loss of the normal AKR/J H-2D^{k} private specificity was detected with a concomitant gain of an H-2D^{d} specificity.

Significance to Biomedical Research and the Program of the Institute: This project is of basic importance for tumor immunology as well as for the genetic expression of MHC controlled cell surface antigens. These findings raise the possibilities that: (a) tumor viruses may be associated with the regulation and/or deregulation of cell surface antigen expression; and (b) an animal strain expressing a particular MHC haplotype may have the genetic information for a number of haplotypes but the expression of a particular haplotype may be influenced by regulator genes mapping within the MHC.

Proposed Course of Project: Testing of the carcinoma cell line for an enzyme marker not linked to H-2 suggested that the origin of the cell line may not have been AKR and may have been of A strain origin. If so, the immunity observed would have been due to an AKR anti-A response, which is allogeneic at the D-end, and therefore would not have been due to a change of H-2D antigens. This project has been terminated.

Publications

Levy, R., Waksal, S. and Shearer, G.: Control of immunity in parental and F_1 hybrid mouse strains to a spontaneously arising parental tumor. I. Correlation between in vivo and in vitro anti-tumor reactivity in responsive and unresponsive animals. Eur. J. Immunol. 7:170-174, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05052-03 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Psychosocial Factors in the Prognosis of Melanoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. N. Rogentine	Senior Investigator	I NCI
OTHER:	J. Rosenblatt	Clinical Associate	AP NCI
	D. P. Van Kammen	Chief, Unit on Neuropsychopharmocology	AP NCI

COOPERATING UNITS (if any)

W. Bunney, NIMH; B. Fox, W. Terry, S. Rosenberg, and R. Fisher, NCI

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20014

TOTAL MANYEARS: 0.85	PROFESSIONAL: 0.85	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the effect of various psychosocial factors on host resistance to malignant melanoma. A detailed characterization of these factors in patients with melanoma, Clinical Stages I and II, is being obtained. In addition certain endocrine and immune parameters are also being studied. We are testing several hypotheses which may distinguish those patients suffering recurrence of their disease from others who either remain disease-free or have a delayed recurrence. At one year, significant correlations with disease-free status in 31 patients were found with (1) number of positive lymph nodes, (2) ABO type, and (3) melanoma adjustment self-rating score (see text). A subsequent group of 33 patients studied confirmed that the number of nodes and the melanoma adjustment score correlated with the one year disease status. Thus it would appear that a psychological variable, the melanoma adjustment score is of major importance in predicting relapse.

Project Description

Objectives: Malignant melanoma is an unpredictable disease. Its course can range from rapid demise to spontaneous remission. The reasons for this variability are not known.

Psychosocial factors are known to play a role in the development of a wide variety of medical illness: e.g. duodenal ulcer and coronary heart disease and there is a growing body of evidence that such factors predispose normal individuals to malignancy, or modify its course once established. It is thus conceivable that psychosocial factors may account for some of the variability in the clinical course of malignant melanoma.

Surgical resection can render melanoma patients disease free. Many of such "cured" patients subsequently develop clinical Stage III disease (distant metastases), usually within two years and go on to die. For a period of time, however, there exists a unique group of patients with known cancer, potentially cured, but at risk of recurrence. Since much previous psychosomatic work has been retrospective in type and fraught with the inherent methodological flaws of that approach, this patient group presents a uniquely important opportunity to carry out a well controlled prospective study on the influence of psychosocial factors on disease progression.

Our purpose is to develop a detailed characterization of and to test several hypotheses regarding the psychosocial factors which distinguish those patients who suffer recurrence of the malignancy from those who either remain disease-free or who have a delayed recurrence.

Methods Employed: We plan to employ the following methods in generating a psychosocial profile of these patients.

1. Psychiatric Interview: Each patient will be seen following surgical recuperation or during clinical staging for an interview of approximately an hour and a half duration. The purpose of this interview will be to gather data regarding the patient's past childhood, occupational and marital history; to identify the relevant psychosocial factors possibly involved in precipitating the current illness; and to make an assessment of the patient's attitude toward the future, his involvement, integrity of ego functions, and object relations.

2. Standardized Questionnaires·

A. MMPI - This is an empirically derived and extensively studied instrument which generates a profile of the patient in several dimensions of personality. This test has very high reliability and will be administered once.

B. Rahe Recent Life Changes Questionnaire - This is a 55-question form used to assess the magnitude of life change the patient has experienced during a given time interval. It has had the benefit of extensive validation study and previous research has demonstrated a high correlation between the magnitude of life change measured by this instrument and the onset of subsequent illness. This scale will be administered initially and at 2-month intervals.

C. SCL-90 Symptom Checklist - This is a self-administered form which checks for the presence of a wide variety of psychological and neurovegetative symptoms. It will be administered initially and at 2-month intervals.

D. Rotter Internal - External Locus of Control Scale - This is a 29-item self-administered form which is currently being widely researched. It appears to be personality dimension with correlates from the neurological to social level of organization. This test also has very high reliability and will be administered only once.

3. Psychobiological Measures: The concurrent intensive study of the immune system being conducted with these patients will also allow us to gather data which may suggest a mediating mechanism for the effect, if any, of psychosocial factors on the progression of the disease. It is possible that psychosocial stress may modify immune responses, for example, via at least two stress responsive hormones, cortisol and catecholamines. Both these hormones modify immune responses. Since the immune system seems to play a role in the development and containment of cancer, psychosocial factors may influence malignancy via the intermediary of the immune system.

In addition to the assays of immune competence we will collect on the initial admission 24-hr urine samples for 17 hydroxycorticosteroid and 17 ketosteroid levels, and VMA excretion.

Once these tests are conducted and scored, the results will be correlated with the patient's stage of disease progression. Our aim is to construct a psychosocial and psychobiological profile of "progression" and "non-progressors".

Major Findings: We have studied two groups of patients: an initial 31 to derive significant associations and a subsequent group of 33 to validate our initial findings. In our first group, relapse of 18 within about one year after surgery for cure was significantly associated with three variables. First, in response to the initial question of the Rahe Recent Life Changes Questionnaire, relapsers weighted on a scale of 1 to 100 the amount of personal adjustment needed to cope with their having melanoma at 52±30. Non-relapsers rated their melanoma adjustment at 83±17, $p<.005$. Secondly relapsers had more positive lymph nodes $p<.05$. This confirms previous studies. Finally, and surprisingly, patients with blood group O tended to relapse (16 of 20), whereas those with blood group A tended to stay in remission (only 2 of 10 relapsed), $p<.01$. Thus, aside from blood group, relapsers either had evidence of extensive nodal metastases or apparently felt their having melanoma did not require as much adjustment to cope with as did non-relapsers. In this group, we distinguished relapse status retrospectively in 29 of 31 patients on the basis of two criteria: relapse occurring (a) in all those having seven or more positive lymph nodes regardless of melanoma adjustment score, and (b) in those having fewer than seven positive nodes if their melanoma adjustment score was less than 65 (the overall mean score).

With a single exception, the second group of 33 patients validated these findings. Eleven had relapsed within about one year after surgery. The melanoma adjustment scores were again different: relapsers = 56±33, non-relapsers = 79±22, $p \cong .01$, 1 tail, t test. Relapsers had significantly more positive lymph nodes, $p \cong .025$, 1 tail, t test. Red cell antigen type did not correlate. Applying the two relapse criteria detailed above, we predicted 9 of 11 relapsers and 16 of 22 remitters, overall accuracy 77% (25/33), $p<.01$, X^2, Yates correction.

Although we are uncertain of the psychodynamic meaning of different melanoma adjustment scores, it appears we are measuring a psychological variable which may be as important as known biological indicators in the short-term prognosis of this malignancy.

Significance to Biomedical Research and the Program of the Institute An understanding of all environmental and host factors in oncogenesis is necessary to progress in cancer research. Psychosocial factors can influence disease occurrence and prognosis. The magnitude of the impact of these factors on host resistance to neoplasms has never been adequately documented, in large part due to the retrospective design of previous clinical experiments. This study avoids that problem: it is a prospective study. Identification of psychological or social stress factors in the promotion of tumor growth is clearly of importance basically as well as for its therapeutic implication. Just as immunotherapy is designed to strengthen host resistance to tumor, psychotherapy could conceivably be used for the same reason.

Proposed Course of Project: We propose to enter about 50 more patients into the study. The data from the first 64 patients will be the basis of hypotheses which the next group of patients will test.

If the correlation between psychosocial variables and disease progression continue as strong, we will consider studying appropriate psychotherapeutic measures in the treatment of melanoma.

In addition any correlations between psychosocial factors and endocrine or immune variables will be sought and appropriate further clinical experiments will be designed to investigate their nature more fully.

We would like to expand this area of endeavor to include other malignancies with short prognoses, such as lung cancer.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05053-03 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Resistance to AKR Leukemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. M. Zatz	IPA Investigator	I NCI
OTHER:	A.-M. Schmitt-Verhulst	Expert	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: .25	PROFESSIONAL: .25	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(AKR/JxDBA/2J)F_1 and H-2 compatible AKR/Cu mice resist transplantation of spontaneous AKR/J lymphoma. Resistance correlates with the development of cell mediated lympholysis (CML) in vitro. A primary F_1 anti-AKR CML response results in Thy^+ effector cells which lyse normal or lymphoma cells, suggesting that parental rather than tumor antigens are involved. The results suggest that "Hh" antigens are expressed on, and play a role in rejection of, spontaneously arising lymphoma cells. In vitro CML response between AKR substrains occurs only after in vivo priming with normal or lymphoma cells. The secondary in vitro CML can be elecited with, and assayed on, normal or tumor cells. Although the only known antigenic difference between the AKR substrains is the Thy antigen, B and T cells are equally good as immunogens and targets. Thus, the CML response is not directed solely to the Thy antigen. This CML is H-2 restricted for responder, immunogen and target cells. The data suggest the existence of a minor H antigen linked to the Thy locus. The AKR substrains are only known to differ genetically at a region of chromosome 9, express high titers of murine leukemia virus and have 80-90% incidence of spontaneous lymphoma, yet a limited and possibly definable antigenic modification results in rejection of the reciprocal strains lymphoma.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project are to define the basis for resistance or susceptibility to growth of AKR lymphoma cells in the high leukemia incidence strain of AKR/J mice, F_1 hybrids of AKR/J, and H-2k compatible mice.

Methods Employed:

1. In vivo resistance. The growth of AKR/J lymphoma cells in AKR/J AKD_2F_1/J, AKR/Cu and CBA/J mice were studied. Doses of 10^3-10^6 lymphoma cells from spleen or thymus of AKR/J mice with spontaneous lymphocytic leukemia were injected i.v. into the recipients. Survival was compared between groups. Resistance of AKR/J mice to transplantation of spontaneously arising AKR/Cu lymphoma cells was also investigated.

2. In vitro resistance. Spleens of normal young mice, or of mice previously challenged with AKR/J lymphoma cells, were assayed in vitro for cell-mediated lympholysis (CML). Cells were cultured in vitro for 5 days with appropriate ratios of 2000R irradiated cell immunogens. After 5 days the cells were harvested and assayed for their ability to kill ^{51}Cr labelled tumor targets (RDM4, derived from AKR/J) or appropriate PHA-stimulated spleen lymphocytes.

Major Findings:

1. AKD_2F_1 resistance. $AKRxDBA_2$ (H-2k, Thy 1.1xH-2d, Thy1.2)F_1 hybrids show selective resistance to growth of AKR/J (H-2k/Thy 1.1) lymphoma cells at doses of 10^3-10^5 cells. Survival is prolonged anywhere from 1 week to 1 month, depending on the experiment and dose employed; eventually AKD_2F_1 mice develop lymphoma and die.

2. AKR/Cu resistance. AKR/Cu mice, derived from the same common stock as AKR/J, have a mutation of chromosome 9, rendering them Thy 1.2 instead of Thy 1.1. Other changes are associated with this mutation, i.e., a difference in an esterase enzyme. These AKR/Cu mice, which resemble the AKR/J mice in their high titer of murine leukemia virus and spontaneous incidence of lymphomas, are resistant to growth of AKR/J tumor cells. At doses of 10^5 cells, 100% of mice survive the injection indefinitely; at doses of 10^6 cells, 80% of mice survive. The reciprocal experiment gives similar results, i.e., AKR/J mice survive indefinitely the injection of 10^5 AKR/Cu lymphoma cells.

3. CBA resistance. CBA mice which are H-2k, but Thy 1.2, also differ from AKR/J mice at multiple minor histocompatibility loci. These mice are even more resistant than are AKR/Cu mice to growth of AKR/J lymphoma. Survival of mice with injection of $5x10^6$ AKR/J lymphoma cells is 100%.

4. AKD_2F_1 anti-AKR/J CML response. AKD_2F_1 cells can be sensitized in vitro by normal and lymphoma cells. The responder cells can be inactivated by prior in vivo silica treatment; effector cells can be inactivated in vitro by anti-Thy serum treatment. The ontogeny of the F_1 anti-AKR/J response is later than

the development of responsiveness to alloantigens. Lysis of RDM_4(AKR/J) tumor cells by F_1 cells sensitized to AKR/J cells in vitro, is inhibited by a H-2k blocking cells (B10.k, C3H, CBA, AKR/J) but not by cells which are not H-2k (B10, B10.D2, DBA or H-2k) for only a portion of the MHC (B10.A, B10.AKM, C3H.OH). These data reveal a K and D end restriction of the CML response. Thus the in vitro CML response resembles other known F_1 antiparent "Hh" responses and may reflect the in vivo basis of F_1 resistance to growth of parental lymphoma cells.

5. CML responses between AKR substrains. Secondary CML responses between AKR substrains occur following priming in vivo with normal or lymphoma cells. Normal lymphoma cells serve equally well as secondary immunogens in vitro and as target cells. Although the only known antigenic difference beween AKR/J (Thy 1.1) and AKR/Cu (Thy 1.2) substrains is the Thy antigen, the present studies demonstrate that the in vitro CML is not related solely to Thy antigen expression. Thus T cells and B cells, as well as spleen and thymus cells, are equally effective as immunogens and targets. Primed AKR/J responder cells will be stimulated by and lyse C3H and B10.BR (H-2k, Thy 1.2) cells, but not CBA cells (H-2k, Thy 1.2), while primed AKR/Cu responder cells will be stimulated by and lyse CBA and B10.BR cells, but not C3H cells. These data, as well as skin graft rejection data, suggest that the in vitro responses between AKR/Cu and AKR/J mice are due to the presence of a minor histocompatibility antigen, previously unknown, which maps in the 9th chromosome.

Further studies show that the expression of the CML response is restricted by the H-2 type of immunogen and target cells. Thus primed AKR/Cu cells require immunogen and target cell homology at the D end of the MHC, while primed AKR/J responder cells require homology at either the D or K end of the MHC.

Significance to Biomedical Research and the Program of the Institute: The AKR/J mouse provides a model for study of acute lymphocytic leukemias of T-cell origin; possibly similar T-cell leukemias, presenting with thymoma, have been described in man. The question of leukemogenesis in AKR/J mice is complex. Several factors play critical roles in determining the onset and incidence of murine leukemia. These factors are 1) the levels of murine leukemia virus, 2) the H-2 type, and 3) the thymus epithelium. It is hoped that by comparing the resistance of the AKR/J mouse to related strains an understanding of the genetic basis of resistance to lymphoma may be achieved. These studies also offer the opportunity to pinpoint antigenic differences, expressed on lymphoma cells, which can elicit effective host immunity.

Proposed Course of Project: Since the principal investigator on this project has left the NIH, this project has been terminated.

Publications

Zatz, M. M.: Resistance to transplantation of spontaneous AKR lymphoma cells by substrains of AKR mice. In vivo resistance and generation of an H-2 restricted CML response. Cell. Immunol. 36: 251-262, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05055-03 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Regulatory Influences on Cell-Mediated Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Head, Immunotherapy Section	I NCI
OTHER:	G. B. Ahmann	Clinical Associate	I NCI
	D. H. Sachs	Head, Transplantation Biology Section	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous investigations have described both antigen-specific and antigen-nonspecific T cell-mediated regulatory mechanisms capable of suppressing the in vitro generation of cell-mediated cytotoxicity. Further experiments have been directed at determining the cellular interactions involved in the in vitro generation of suppressor cells, and at analyzing genetic differences in the abilities of different inbred mouse strains to generate suppressor cells.

Project Description

Objectives: In addition to the effector cells generated in response to antigen stimulus, there has been increasing data presented to suggest that cell-mediated regulatory influences are also activated by antigenic stimulus. It has been the object of these studies to define the regulating influence governing the generation of T cell-mediated responses.

Methods Employed: T cell-dependent suppression of cell-mediated lympholysis was generated by in vitro culture of normal murine spleen cells.

I. The cellular requirements for generation of T cell-dependent suppression were studied by: 1) examining the effect of pretreating normal spleen cells with anti-Ia reagents and C on their ability to generate suppressors, and 2) fractionating spleen cells by passage over Sephadex G-10 columns and analyzing the suppressor activity in column-passed populations.

II. The ability of different inbred mouse strains to generate suppressor activity in vitro was compared.

Major Findings:

I. Cellular requirements for culture-induced generation of suppressor cells. The pretreatment of normal spleen cells with anti-Ia reagents and C abrogated the ability of these cells to generate suppressors during culture, although the treated cells were still capable of themselves giving alloantigen-induced cell-mediated cytotoxicity. In contrast, treating cells with anti-Ia and C after they had developed suppressor activity was without effect. These findings established a requirement for an Ia positive population which participates in the generation of suppressor cells. Sephadex G-10 passed spleen cells were also unable to generate suppressor activity. In these experiments, it was found that the ability to generate suppressors was reconstituted by the addition of small numbers of splenic adherent, radiation-resistant non-T cells. It is currently under investigation as to whether this non-T cell is identical to, or distinct from, the Ia positive cell independently shown to be required for cell induction.

II. Strain differences in the ability to generate suppressors. BALB/c ($H-2^d$) and B10 ($H-2^d$) spleen cells generated significantly more active suppressor populations than B10.D2 ($H-2^d$) spleen cells. In mixing experiments, it was found that BALB/c suppressors were efficient in suppressing the cell-mediated cytotoxic response of B10.D2 cells, while B10.D2 suppressors were not effective at suppressing BALB/c responses. The defect thus appears to be in the ability of B10.D2 cells to suppress, rather than in a difference in susceptibility to suppression.

Significance to Biomedical Research and the Program of the Institute: States of *in vivo* tolerance or immune hyporesponsiveness have been associated with active suppressive populations in a number of systems. In addition, the possible role of specific or nonspecific immune suppression in the evaluation or progression of malignancy has been suggested. The studies described above may provide information concerning the mechanism of such in vivo immunosuppression and possible means of modifying the suppression as a therapeutic manipulation of the immune response.

Proposed Course: Further studies are in progress to determine the relationship between the Ia positive cell and the adherent non-T cell participating in the generation of suppression. The genetic basis for the observed strain difference in suppressor generation is under study.

Publications

Nadler, L. M. and Hodes, R. J.: Regulatory mechanisms in cell-mediated immune responses. II. Comparison of culture-induced and alloantigen-induced suppressor cells in MLR and CML. J. Immunol. 118: 1886-1895, 1977.

Hodes, R. J., Nadler, L. M. and Hathcock, K. S.: Regulatory mechanism in cell-mediated immune responses. III. Antigen-specific and nonspecific suppressor activities generated during MLC. J. Immunol. 119: 961-967, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-05056-03 I

PERIOD COVERED October 1, 1977 to June 30, 1978

TITLE OF PROJECT (80 characters or less)

Specific Removal of Histocompatibility Antigen-Reactive Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Neefe, Jr.	Investigator	I	NCI
OTHER:	D. H. Sachs	Head, Transplantation Biology Section	I	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Receptors for transplantation antigens may be visualized directly on unsensitized cells with the use of an anti-idiotypic antisera. It should be possible to eliminate these cells specifically by treatment of the unsensitized population with sera directed at the specific binding site. Sera have been raised to cells cytotoxic for transplantation alloantigens in strain combinations selected so that only the variable portion of the antigen specific receptor could be recognized. Bonafide anti-idiotypic sera have not been raised. Refinements in the approach under way include 1) use of cytotoxic populations as immunogen with a demonstrably high proportion of receptor bearing cells, 2) use of parent strains differing only by a point mutation to restrict the heterogeneity of the immune response of one against the other, and 3) use of continuously cultured cytotoxic cell lines to increase the homogeneity of the receptors used as immunogen.

Project Description

Objectives: Several lines of evidence indicate clonal diversity of T lymphocytes reactive to transplantation antigens, and recently it has been possible to visualize directly receptor-bearing lymphocytes in unsensitized populations. If precursors of cells reactive to transplantation antigens bear receptors to those antigens, it should be possible to render a lymphoid population tolerant of those antigens by inhibition of the receptor-bearing cells using antibody with specificity for the binding site of the receptor (anti-idiotypic antibody).

In addition to the obvious usefulness of such techniques and principles for study of the generation of cellular immunity, some clinical applications are of importance, as in specific tumor immunotherapy. Allogeneic cells sensitized by whatever means against tumor-associated antigens could prevent the appearance or recurrence of tumor, but they must also be rendered incapable of causing graft-vs-host disease by elimination of any capacity to react to the transplantation antigens of the host.

Methods Employed: Studies were performed using normal mouse spleen cells. Reactivity to transplantation antigens was assessed at the effector stage by the capacity to generate specifically cytotoxic cells in mixed lymphocyte culture. Anti-idiotypic antibodies were generated by multiple immunization of F_1 hybrid mice with cytotoxic cells of one parent strain sensitized to transplantation antigens of the other parent. Cytotoxic cells were enumerated as a proportion of a sensitized population in a limiting dilution assay using 48 replicates with a small number of very heavily labelled targets per well.

Major Findings: Putative anti-idiotype sera have been raised in several strain combinations and tested for their ability to lyse, in the presence of complement, precursors of killer cells or killer cells themselves. To date no bonafide anti-idiotypic serum has been prepared.

Refinements in the approach have been developed. Parent strains differing only in a few amino acids of one of their major histocompatibility antigens have been employed to reduce the heterogeneity of the idiotypes generated. Efforts have been directed at generating populations of immune cells in this combination with a high proportion of receptor bearing cells. It has been possible to show that cytotoxic cells generated in vitro with a secondary boost in vitro contain at least 10% immunologically specific and therefore receptor-bearing killer cells and sometimes more than 20%. Use of such populations as a source of idiotype for immunization to produce anti-idiotypic sera is in progress.

Significance to Biomedical Research and the Program of the Institute: Transplantation may be useful for cancer therapy in several settings. Transplants of lymphoid cells appropriately sensitized may control the appearance or growth of tumors. Transplants of organs may replace organs ravaged by tumors. Rescue with transplants of bone marrow may permit use of doses of anticancer chemotherapy which would otherwise be lethal. In each setting, success depends in part on specific control of responses to histocompatibility antigens. This project examines one means for eliminating these responses specifically.

Proposed Course of Project: Testing of sera described above for anti-idiotypic properties will continue. Recently published methods for developing continuously cultured cytotoxic T cell lines will be explored as a possible means for increasing receptor homogeneity. The Principal Investigator is terminating June 30, 1978; any future work will be carried on at his new place of employment outside of NIH.

Publications

Gensheimer, G. G. and Neefe, J. R.: Cell-mediated lympholysis: A receptor-associated lytic mechanisms. Cell. Immunol. 36: 54-64, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05058-03 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunoregulation by Antibody

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Pincus	Investigator	I NCI
OTHER:	H. B. Dickler	Senior Investigator	I NCI
	C. O. Cowing	IPA Investigator	I NCI
	A. Singer	Investigator	I NCI
	R. J. Hodes	Head, Immunotherapy Section	I NCI
	D. H. Sachs	Head, Transplantation Biology Section	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.675	PROFESSIONAL: 1.375	OTHER: 0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies specific for the idiotype(s) of anti-(T,G)-A--L antibodies of two different mouse strains (C57BL/10Sn and C3H.SW) have been obtained. The idiotype(s) of these two strains are distinct and the anti-(T,G)-A--L antibodies of the majority of individuals of each strain express identical or cross-reactive idiotype(s). Since the ability of mice to respond to (T,G)-A--L is under the control of H-2 linked Ir genes, these anti-idiotype antibodies are being used in conjunction with radioimmunoassay of the binding of (T,G)-A--L to anti-(T,G)-A--L antibodies, antigen-specific T lymphocyte proliferation to this antigen, and the in vitro primary antibody response to TNP-(T,G)-A--L to evaluate the cellular and genetic control of idiotype expression as well as the role of idiotype-bearing molecules in regulation of the immune response. T lymphocytes express anti-(T,G)-A--L idiotype(s) identical to or cross-reactive with those found on anti-(T,G)-A--L antibodies of the same strain.

Project Description

Objectives:

1. Determine the genetic control of idiotype expression on anti-(T,G)-A--L antibodies.

2. Determine the immunocompetent cell types which express idiotype bearing molecules (receptors).

3. Evaluate the role of idiotype-bearing molecules in regulation of the immune response.

Methods Employed:

1. Preparation of anti-idiotypic antisera by immunization of Lewis rats with antigen affinity purified murine anti-(T,G)-A--L antibodies and adsorbtion on normal mouse globulins.

2. A radioimmunoassay that measures the binding of radiolabelled (T,G)-A--L by anti-(T,G)-A--L antibody. Anti-idiotypic activity is measured as inhibition of (T,G)-A--L binding.

3. Immunization of mice of different strains, including congenic resistant and recombinant inbred lines, with (T,G)-A--L and determining via radioimmunoassay which mice bear idiotypes defined by these antisera.

4. Antigen specific primary *in vitro* humoral responses of mouse spleen cells to the antigen (T,G)-A--L-TNP.

5. Antigen-specific secondary T cell proliferation of mouse lymph node cells to (T,G)-A--L.

Major Findings:

1. The following antisera have been produced: Lewis anti-[C3H.SW anti-(T,G)-A--L] and Lewis anti-[B10 anti-(T,G)-A--L]. After adsorbtion on normal globulin from C3H.SW and B10 mice, respectively, the antisera have been shown to be directed against idiotypic determinants by a variety of criteria. The idiotype(s) of these two strains are distinct, and the anti-(T,G)-A--L antibody of the majority of individuals of each strain express identical or cross-reactive idiotype(s).

2. A T cell proliferation assay, using (T,G)-A--L as the antigen has been developed. Macrophages pulsed with high concentrations of (T,G)-A--L are used to present the antigen to (T,G)-A--L primed nylon wool purified mouse lymph node cells.

3. Using a previously described primary in vitro humoral response assay, it has been found that the Lew anti-[B10 anti-(T,G)-A--L] sera inhibits the

response to (T,G)-A--L-TNP but not KLH-TNP in B10 mice, but not in C3H.SW mice. Also, treatment of T lymphocytes with anti-idiotype antibodies and complement abrogates the ability of such cells to cooperate with B lymphocytes and accessory cells in the response to (T,G)-A--L-TNP.

Significance to Biomedical Research and the Program of the Institute: Since idiotypes define a single or small number of clones of antibody, the determination of the genetic control of idiotype expression bears directly on the origins of diversity of antibody. An understanding of the genetic and cellular control of expression of idiotype bearing molecules (including antibody and T lymphocyte receptors) will clarify the role of such molecules in regulation of the immune response. An understanding of the regulation of the immune response could lead to new forms of therapy for human diseases including cancer.

Proposed Course of Project:

1. Map the genes which determine expression of anti-(T,G)-A--L idiotype(s) on antibodies.

2. Evaluate T lymphocytes and accessory cells for expression of anti-(T,G)-A--L idiotype(s).

3. Map the genes which determine expression of anti-(T,G)-A--L idiotype(s) on T lymphocytes (? and accessory cells).

4. Evaluate the role of other cell types (e.g. thymic epithelium) in the expression of idiotypes by T lymphocytes.

5. Isolate idiotype bearing molecules from T lymphocytes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05059-03 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Characterization of Antigen Specific T-Cell Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Singer	Investigator	I NCI
OTHER:	H. B. Dickler	Senior Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antigen specific T-cell factors are most likely the solubilized T-cell antigen receptor, since it is unlikely that the T-cell has two different antigen specific molecules, the factor and a different receptor. Therefore, antigen specific T-cell factors offer a direct way to study the structure of the T-cell antigen receptor. In addition, the immunoregulatory functions of these factors provide a way of studying specific B-cell activation and T-B cooperation. Although such factors were shown to be active in vivo, preliminary experiments indicate that are not active in vitro.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The three major objectives of this project are (1) the confirmation of the existence of antigen specific T-cell factors, (2) the physical characterization of such factors, and (3) the study of the immunoregulatory function of antigen specific T-cell factors in T-B and T-T cooperation.

Methods Employed: Thymocytes from C57BL/10 mice educated to TGAL are cultured with TGAL for 6-8 hours under serum-free conditions. The supernatants from these cultures contain an antigen specific T-cell product which can replace T-cells in an adoptive transfer experiment. The biologic activity of this factor is assayed by adoptively transferring into a syngeneic animal either bone marrow cells and TGAL, bone marrow cells plus educated thymocytes and TGAL, or bone marrow cells plus the supernatant factor and TGAL. The development of specific antibody producing cells in the spleens of the transferred animals is assayed by the Jerne plaque technique. However, such factors could be best characterized in the in vitro antibody response system recently developed (see Project No. Z01-CB-05064-02-I). The following methods are used to characterize the factor:

(a) the use of genetically different strains of mice to produce and respond to the factor,
(b) column purification of the factor.
(c) titration of varying amounts of factor with B-cells,
(d) gel filtration to determine molecular weight,
(e) absorption of factor by anti-Ia, antigen, anti-immunoglobulin, and anti-idiotype immunoadsorbents.

Major Findings: TGAL specific T-cell factor can be obtained from educated thymocytes cultured with TGAL under serum-free conditions for 6-8 hours. This factor can cooperate with normal bone marrow cells and TGAL to induce the development of cells which secrete specific anti-TGAL antibody in the absence of T cells. The factor produced by TGAL educated thymocytes is antigen specific and does not cooperate with B-cells to form anti-SRBC antibodies. Unfortunately such factors do not appear to be active in in vitro antibody-forming cell assays.

Significance to Biomedical Research and the Program of the Institute: T-cells are one type of lymphoid cell involved in tumor recognition and destruction, yet the T-cell antigen recognition system is poorly understood. Greater insight into the structure and function of the T-cell antigen receptor will likely lead to a deeper understanding of the roles T-cells and their products play in tumor immunology. Furthermore, antigen specific T-cell factors can function as immunoregulatory molecules either enhancing or suppressing antigen specific immune responses. They offer a potentially potent tool for manipulating the immune response against specific targets.

Proposed Course of Project: Since such factors are not active in vitro, it becomes difficult to accomplish the objectives of this project. Thus, the project is being terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05060-03 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Physiological Role of Lymphocyte Surface Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Henkart	Senior Investigator	I NCI
OTHER:	J. L. Ryan	Investigator	I NCI
	M. Shinitzky	Visiting Scientist	I NCI
	S. Ben-Sasson	Visiting Fellow	I NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Immunology Branch

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Intracellular levels of cAMP and cGMP were measured by radioimmunoassay in mouse spleen cells settled onto surfaces coated by antigen-antibody complexes or $F(ab')_2$ complexes in the presence or absence of a mitogenic concentration of LPS. cAMP levels were consistently elevated (1.5x) after 30 minutes of contact with immobilized complexes; however, $F(ab')_2$ complexes did not induce this change, nor did LPS affect the cAMP levels. No significant change in cGMP levels was found to be induced by immobilized complexes.

Some preparations of gangliosides were found to be mitogenic for mouse spleen cells. Purified ganglioside fractions which were non-immunogenic inhibited LPS-induced mitogenesis but not PHS induced mitogenesis.

MLC type reactions between lymphocytes and tumor cells were shown to be enhanced by inclusion of dextran and polyethyleneglycol in the culture medium. These results were interpreted as being due to enhanced lymphocyte-tumor interaction via specific receptors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To define the physiological function of the Fc receptor. To test the hypothesis that lymphocyte glycolipids may play a physiological role in the immune response. To attempt to enhance weak lymphocyte-tumor cell interactions.

Methods Employed: Mouse spleen lymphocytes were allowed to settle on petri plates coated with TNP protein with and without anti-TNP. After various times the supernatant and cells were collected, treated with TCA and the cyclic nucleotide levels measured by radioimmunoassay after separation by ion exchange chromatography. Gangliosides from bovine brain were purchased and further fractionated on TLC. Standard culture conditions were employed for MLC and mitogenesis experiments.

Major Findings: Intracellular levels of cAMP and cCMP were measured by radioimmunoassay in mouse spleen cells settled onto surfaces coated by antigen-antibody complexes of $F(ab')_2$ complexes in the presence or absence of a mitogenic concentration of LPS. cAMP levels were consistently elevated (1.5x) after 30 minutes of contact with immobilized complexes· however, $F(ab')_2$ complexes did not induce this change. nor did LPS affect the cAMP levels. No significant change in cGMP levels were found to be induced by immobilized complexes.

The commercial ganglioside preparations which are mitogenically active were further fractionated by precipitation. The active fraction is less chloroform soluble than most gangliosides. It contains protein and sugar and therefore the activity may be a contaminant rather than a glycolipid. The specifically suppressive ganglioside fractions appear to coincide with the major classes of gangliosides in cells. It is hypothesized that these gangliosides may compete with LPS for binding sites in the membrane.

Enhanced thymidine incorporation due to an MLC type reaction was observed when the hydrophilic polymers dextran and PEG were present in the medium during culture of lymphocytes and syngeneic tumor cells. Similar enhancement was observed for classical MLC reactions done at suboptimal conditions. The polymers alone are not mitogenic.

Significance to Biomedical Research and the Program of the Institute: The increased cAMP levels mediated by lymphocyte Fc receptors confirms our proposal on the mechanism of the inhibitory effects previously observed. The ganglioside experiments provide evidence that naturally occurring cell surface molecules can modulate the immune response. Finally enhanced in vitro responsiveness of lymphocytes to tumor cells may be important in developing techniques for enhancing immunity to tumors.

Proposed Course of Project: This is the conclusion of this project since most of the investigators are no longer working at the National Institutes of Health. Two papers have been submitted for publication.

Publications

Ben-Sasson, S. and Henkart, P.A.: Enhancement of weak MLC type reactions by hydrophilic polymers. J. Immunol. 119: 227-231, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05062-03 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Application of Rapid Flow Microfluorometry to Cell Biology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
	S. O. Sharrow	Chemist	I NCI
OTHER:	T. M. Chused	Medical Officer (Research)	LI NIAID
	D. H. Sachs	Head, Transplantation Biology Section	I NCI
	P. A. Henkart	Senior Investigator	I NCI
	J. N. Weinstein	Investigator	LTB NCI
	H. C. Morse	Senior Investigator	LMI NIAID
	L. D. Leserman	Clinical Associate	I NCI
	W. D. Terry	Assoc. Dir., IP	I NCI
	H. M. Moutsopoulos	Visiting Associate	A&R NIAMDD
	W. F. Davidson		
	B. J. Mathieson	Staff Fellow	LMI NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using rapid-flow microfluorometry for analysis and sorting of cells, aspects of the following projects have been supported during the previous year: (1) expression of I and Ly antigens on thymocytes; (2) regulatory gene control of expression of cell surface antigens coded for by xenotropic murine leukemia virus genomes; (3) characterization of liposome-cell interactions; and (4) early B lymphocyte abnormalities in autoimmune mice.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objective of this project is to utilize rapid flow microflourometry (FMF) to study selected aspects of important problems which would be impossible or extremely difficult to pursue without such technical support.

Methods Employed: Cells have been both analyzed and separated by rapid flow microfluorometry (Fluorescent-Activated Cell Sorter (FACS), Becton-Dickinson Electronics Laboratory, Mountain View, CA). These studies involve preliminary treatment of suspensions of viable, dispersed cells with purified, well-characterized fluorescent reagents.

Major Findings: This report summarizes the major thrust of each overall project, emphasizing those aspects most heavily supported by use of rapid flow microfluorometry (FMF).

Murine thymocytes have been examined by FMF for two classes of cell surface antigens which appear to be related to cell differentiation and function. Work with Dr. Mathieson has revealed that previous reports on the distribution of thymocyte differentiation antigens, Lyt 1,2,3, on thymocytes are misleading. Use of FMF with highly specific antisera shows that approximately 10% of thymocytes express only Lyt 1 as opposed to previous reports that all three phenotypes are expressed on all individual thymocytes; this finding has a significant bearing on the state of differentiation of thymocytes. Lyt 1 phenotype expression is increased on cortisone resistant thymocytes in contrast to decreased amounts which would be predicted from previous data. Discrepancies between the current findings and previous reports are most likely attributable to the greater sensitivity and resolution of FMF analysis. Additional studies with Dr. Sachs show that Ia antigens, products of genetic regions which regulate immune responses to thymus dependent antigens, are clearly expressed on at least 50% of normal thymocytes. The reliability of these findings was enhanced by using antisera made between congenic mouse strains and by testing thymus cells from congenic strains in order to confine reactions more to Ia determinants. Reaction patterns indicated that I antigens from more than one I subregion are expressed on thymus cells and that these antigens, unlike those detected by rabbit anti-mouse brain antisera and anti Lyt antisera, are not associated with thymocyte subpopulations distinguished by cell size.

Earlier work on liposome-cell interactions, reactions of interest as a model for virus infections and as a means for introducing membrane impermeable substances into cells, has continued with Drs. Weinstein, Blumenthal, Henkart, Leserman and Terry. Analysis of kinetic data suggests that there are two pathways for transfer of liposome contents into nonphagocytic cells (e.g. lymphocytes), one involving a saturable site on the surface of the target cell, the other, either a separate, less easily saturable site or an endocytosis process not involving a specific cell surface site. Attempts to target or direct liposomes to specific cells by use of antibody bridging between chemically modified (TNP) cells and liposomes has been achieved. However, vesicle contents were not delivered intracellularly. This failure has been attributed to a) attaching liposomes to inappropriate sites on the cell surface, b) spacing liposomes too far from the cell surface because of intervening antibody or c) antibody induced aggregation of liposomes at the cell surface. These findings have been confirmed using a mouse myeloma tumor naturally bearing surface immunoglobulin which reacts with a chemical determinant, DNP. Liposomes, treated with DNP, specifically bound to the myeloma cells; but again binding did not result in increased intracellular delivery of the vesicle contents.

A mouse strain with a particularly high incidence of autoimmune disease, NZB, has been widely said to show a loss of suppressor cells prior to onset of abnormalities associated with autoimmunity. FMF studies with Drs. Chused and Moutsopoulos show that cytoplasmic Ig abnormalities of B lymphocytes occur before known loss of suppressor T cells. Thus spleen cells from NZB mice have an abnormally increased percentage of cells with cytoplasmic immunoglobulin by two weeks of age. These findings are consistent with earlier reported radio-immunoassays showing that IgM secretion by spleen cells from NZB s is abnormally high, even at birth, and that IgM secretion per cell was not influenced by FMF removal of T cells.

Earlier FMF investigations of cell surface antigens coded for by endogenous genes for xenotropic murine leukemia virus (Xen CSA) indicated that expression of the antigens in breeding crosses between DBA/2 and C57BL/6 mice is under the control of a regulatory gene linked to or associated with the Fv-1 locus, which controls susceptibility of cells to infection by ecotropic viruses. Additional strain combinations (DBA/2 and C57L) have now been examined and these too show regulatory gene control of Xen CSA expression. This locus, however, is not linked to Fv-1. Complexicity of Xen CSA regulation is further demonstrated by analysis of a series of H-1 congenic mice on a C57BL/10 background; Xen CSA expression correlated with H-1 in certain strains, suggesting an H-1 associated influence.

Significance to Biomedical Research and Program of the Institute: I antigens are products of a genetic region which controls immune responses to T-dependent antigens among which may be tumor associated antigens. Identifying the subclasses of thymus derived cells in which these genes are normally activated is thus an important link in understanding the immune response to tumor cells.

Understanding the process of liposome-cell interactions is important because this process offers a model for virus penetration of host cells and is among the most promising means for introducing membrane impermeable substances into cells and as such is important for developmental chemotherapy and experimental cell transformation.

The heterogeneity of endogenous MuLV gene products on the surfaces of normal and neoplastic cells has only recently become apparent. This large family of cell surface components is postulated to have a role in normal cell differentiation and in regulation of tumor growth. Investigating genetic regulation of expression of MuLV gene products on cell surfaces is thus an important step in understanding the control of cell growth and differentiation.

Proposed Course of Project: As in the past, rapid flow microfluorometry will be used for selected, appropriate projects.

Publications

Schwartz, B. D., Kask, A. M., Sharrow, S. O., David, C. S., and Schwartz, R. H.: Partial chemical characterization of Ia antigens derived from murine thymocytes. Proc. Natl. Sci. USA 74:1195-1199, 1977.

Slater, M. L., Sharrow, S. O., and Gart, J. J.: Cell cycle analysis by flow microfluorometry of *Saccharomyces cerevisiae* growing at different rates. Proc. Natl. Acad. Sci. 74:3850-3854, 1977.

Campbell, G. LeM., Schachner, M., and Sharrow, S. O.: Isolation of glial cell-enriched and -depleted populations from mouse cerebellum by density gradient centrifugation and electronic cell sorting. Brain Res. 127: 69-86, 1977.

Blumenthal, R., Weinstein, J. N., Sharrow, S. O., and Henkart, P.: Liposome-lymphocyte interaction: Saturable sites for transfer and intracellular release of liposome contents. Proc. Natl. Acad. Sci. USA 74: 5703-5604, 1977.

Scher, I., Ahmed, A., and Sharrow, S.O.: Murine B-lymphocyte heterogeneity: Distribution of complement receptor-bearing and minor lymphocyte stimulating B lymphocytes among cells with different densities of total surface Ig and IgM. J. Immunol. 119: 1938-1942, 1977.

Scher, I., Ahmed, A., Sharrow, S., and Sell, K. W.: The murine minor lymphocyte stimulating locus defines a B cell subclass whose expression is dependent on genes on the X chromosome. Transplant. Proc. 9: 813-817, 1977.

Scher, I., Ahmed, A., Sharrow, S. O., and Paul, W. E.: Maturation of murine B lymphocytes. In Cooper, M. D. and Dayton, D. H. (Eds.) *Development of Host Defenses*. New York, Raven Press, 1977, pp. 44-74.

Dickler, H. B., Kubicek, M. T., Arbeit, R. D., and Sharrow, S. O.: The association between Ia antigens and Fc receptors on murine B lymphocytes is probably ligand-induced. In McDevitt. H. L. (Ed.), *Ir Genes and Ia Antigens*. New York, Academic Press, Inc., 1978, pp. 185-193.

Dickler, H. B., Kubicek, M. T., Arbeit, R. D., and Sharrow, S. O.: Studies on the nature of the relationship between Ia antigens and Fc receptors on murine B lymphocytes. J. Immunology. 119:348-354, 1977.

Miller, M. H., Powell, J. I., Sharrow, S. O., and Schultz, A. R.: Rapid data collection, analysis and graphics for flow microfluorometry instrumentation. Rev. Sci. Instrum., in press.

Weinstein, J. N., Blumenthal, R., Sharrow, S. O., and Henkart, P. A.: Antibody-mediated targeting of liposomes: Binding to lymphocytes without incorporation of vesical contents into the cells. Biochim. Biophys Acta, in press.

The following are publications in which FMF technology has made a contribution but was not the major technique utilized:

Moutsopoulos, H. M., Boehm-Truitt, M., Kassan, S. and Chused, T. M.: Demonstration of activation of B lymphocytes in New Zealand black mice at birth by an immunoradiometric assay for murine IgM. J. Immunol. 119: 1639-1644, 1977.

Henkart, P. A., Schmitt-Verhulst, A.-M., Shearer, G. M.: Specificity of cytotoxic effector cells directed against trinitrobenzene sulfonate-modified syngeneic cells. Failure to recognize cell surface-bound trinitrophenyl dextran. J. Exp. Med. 146:1068-1078, 1977.

Singer, A. Cowing, C., Hathcock, K. S., Dickler, H. B., Hodes, R. J.: Cellular and genetic control of antibody responses in vitro. III. Immune response gene regulation of accessory cell function. J. Exp. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-05063-02 I

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Idiotype Specific T-Cell Responder

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lee D. Leserman	Clinical Associate	I NCI
OTHER:	William D. Terry	Associate Director for Immunology	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro assays for studying specific cell-mediated immunity to plasma cell tumors of mice have been established. Antisera directed against the idiotypic antigens of myeloma proteins produced by these tumors have been produced. These systems will now be used to study T-cell recognition of idiotypic antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Lymphoid cells of mice can be sensitized to syngeneic tumor cells *in vitro*, and will produce cytotoxic effector cells which can kill those tumor cells. Despite this apparent ability to elicit an effective immune response, these tumors will grow and kill a syngeneic host following transplantation with little or no evidence of an immune response against them.

Experiments which purport to study responses to tumor specific antigens are difficult to interpret because of the problem of reaction to viral antigens or derepressed fetal antigens, perhaps not unique to the tumor or relevant to tumor antigens present *in vivo*.

In order to follow the response to antigens known to be present on and produced by tumors, we study the response of BALB/c mice to myeloma tumors of BALB origin. It is known that these tumors bear idiotypic determinants which will elicit T-helper and B-cell responses.

Long range goals of this project are (1) to find whether cytotoxic T-cells recognize idiotypic determinants; (2) to see whether immunologic manipulations can increase the representation of cells sensitive to these idiotypes; (3) to determine if these manipulations can induce protective responses in the host or *in vitro*.

Methods Employed: Cell-meditated immunity is measured by release of ^{51}Cr from target cells incubated *in vitro* with sensitized lymphoid cells. These cells may be sensitized by immunization with tumor or idiotypic determinants, or by passive immunization with anti-idiotypic antibody. Effects on idiotype recognition by immunizing with antigens which are bound by idiotypes of known hapten-binding specificity were measured.

Major Findings: We have evaluated the ability of cytotoxic T-cells to recognize idiotypic determinants on the surface of syngeneic murine myeloma cells. Induction of an immune response to myeloma cells has been accomplished; however, no evidence for recognition of idiotype by cytotoxic T-cells has been obtained.

Proposed Course of Project: Since further experiments were predicated on following expression of this marker as a function of immune response to a specific tumor antigen, the study has been terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05064-02 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Singer	Investigator	I NCI
OTHER:	R. J. Hodes	Head, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 3.7	PROFESSIONAL: 1.8	OTHER: 1.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to more precisely define the nature and function of genes controlling the cellular interactions involved in the antibody response and/or Ir genes controlling response to certain soluble antigens, an in vitro system for generating both primary and secondary antibody responses to soluble antigens has been developed and characterized. Responses in the system are T-cell and accessory cell dependent, and for TNP-(T,G)-A--L and TNP-(H,G)-A--L are clearly under H-2 linked Ir gene control. The function of accessory cells in response to TNP-(T,G)-A--L and TNP-(H,G)-A--L is also regulated by H-2 linked Ir genes encoded within the same subregions of H-2 as the overall in vitro and in vivo responses to these antigens. In addition, it has been found that the generation of specific antibody responses to antigen requires the active recognition by T-cells of H-2 antigens (probably Ia antigens) as "self" that are expressed by accessory cells. The H-2 antigens that are in fact recognized as "self" are not determined by the genotype of the T cells, but rather are determined by the genotype of the thymus in which the T-cells matured.

Project Description

Objectives: The three major objectives of this project are: (1) the determination of which cellular interactions are governed by Ir genes so as to determine the method by which these genes function; (2) the study of the regulation of B-cell function by T cells and macrophages and their soluble products; and (3) the characterization of the T cell receptor for "self" which regulates cellular interactions required for the generation of antibody responses to antigen.

Methods Employed: Normal or carrier primed spleen cells are dispersed into single cell suspensions and put into microcultures with TNP modified antigens (KLH, TGAL, HGAL, GAT, OVA, Nase, etc.) for 4 days. The cells are then harvested and assayed for IgM or IgG anti-TNP antibody-producing cells by the slide modification of the Jerne plaque technique.

Splenic B cells are obtained by treating spleen cells with a T-cell specific rabbit antimouse brain serum and complement. Splenic T cells are obtained by passage over a nylon wool column. Splenic adherent cells are obtained by allowing spleen cells to adhere to glass petri dishes for 2 hours and then discarding the nonadherent cells and collecting the adherent cells by the addition of EDTA. Spleen cells are depleted of macrophages by passage over a G10 sephadex column. Chimeric mice are created by lethally irradiating inbred strains of mice and reconstituting them with syngeneic, semi-syngeneic and allogeneic bone marrow cells.

Major Findings: A primary response to TNP-modified derivatives of a wide variety of antigens, including the syngeneic polypeptides, can be obtained. These in vitro responses are T-cell and accessory cell dependent, and for the case of TGAL and HGAL are under H-2-linked Ir gene control. The function of accessory cells in the responses to TNP-TGAL and TNP-HGAL is also regulated by H-2 linked Ir genes. These same accessory cells express Ia antigens on their surface that are encoded within at least two different I subregions.

For the response to TNP-KLH, it has been found that within an F_1 hybrid there exist two independent classes of T cell receptors for "self" - one specific for the H-2 haplotype of each parent. The H-2 specificity of the T-cell receptors for "self" is directed to recognition of the genotype of the accessory cell and appears not to require active recognition of the genotype of the B cell. Experiments with chimeric mice (parent → F_1 and F_1 → parent) suggests that the T-cell specificity for "self" H-2 expressed on the accessory cell does not derive from the genotype of the T cell but rather derives from the genotype of the thymus in which the T cells matured.

Significance to Biomedical Research and the Program of the Institute: The study of genetic control of cellular interactions required in the immune response has been limited by a lack of in vitro assays in which genetic functions are expressed. The development of such an assay system has been accomplished and provides a major advance in their field. In vitro analysis of gene regulation can lead to a greater understanding of how genes function to regulate the immune response. Such understanding will lead to the ability to purposefully regulate immune responses in human disease.

Proposed Course of Project: The project will be directed toward several immediate goals: a) identification of the cell types which express Ir gene defects in nonresponder mice; b) elucidation of the mechanisms by which *Ir* genes regulate immune responses; and c) characterization of the T cell receptor for "self"--its specificity, and the mechanism by which such specificity is derived.

Publications

Singer, A., Dickler, H. B. and Hodes, R. J.: Cellular and genetic control of antibody responses in vitro. II. Ir gene control of primary IgM responses to trinitrophenyl conjugates of poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys and poly-L-(His,Glu)-poly-D,L-Ala--poly-L-Lys. *J. Exp. Med*. 146: 1096-1107, 1977.

Hodes, R. J. and Singer, A.: Cellular and genetic control of antibody responses in vitro. I. Cellular requirements for the generation of genetically controlled primary IgM response to soluble antigens. *Eur. J. Immunol*. 7: 892-897, 1977.

Singer, A., Cowing, C., Hathcock, K., Dickler, H. B. and Hodes, R. J.: Cellular and genetic control of antibody responses in vitro. III. Immune response gene regulation of accessory cell function. *J. Exp. Med*., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05065-02 I

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunologic Function of Accessory Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. O. Cowing	IPA Investigator	I NCI
OTHER:	H. B. Dickler	Senior Investigator	I NCI
	S. H. Pincus	Investigator	I NCI

(collaboration in part with Project No. Z01-CB-05064-02-I)

COOPERATING UNITS (if any)

B.D. Schwartz, Division of Rheumatology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 62110

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.55	1.25	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to explore the mechanism by which accessory cells process and present antigen to lymphoid cells and how they function in the regulation of the immune response. Recent work under this project has shown that accessory cells which supported antigen specific primed lymph node T cell proliferation were Thy 1.2 negative, radioresistant, glass adherent and expressed both I-A and I-E or C subregion antigens; and, that the ability of spleen adherent cells to function as accessory cells in the in vitro primary humoral responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L was controlled by autosomal dominant genes located in the K or I-A regions of the responder H-2 complex and therefore mapped identically to the Ir genes controlling overall in vitro and in vivo responsiveness to these antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To evaluate the role of Ia antigens on accessory cells in terms of the activation of subsets of B and T lymphocytes. 2) To determine the relationship between accessory cell Ia antigens and the expression of Ir genes in these cells. 3) To explore the mechanisms by which accessory cells take up, "process" and present antigen to lymphoid cells.

Methods: 1) Adherent cells are purified from spleen and peritoneal exudate by adherence to glass and removal or inactivation of other cell types by various means including treatment with anti θ and C' and irradiation. 2) Accessory cells are removed from lymphoid cell populations by passage over Sephadex G10. 3) Ia antigens and other surface markers are detected by immunofluorescence. Ia bearing cells are removed by treatment with alloantisera and C'. The synthesis of Ia antigens is measured by internal labeling with ^{3}H-leucine, followed by solubilization, immunoprecipitation and gel electrophoresis. 4) The immunologic function of accessory cells is assessed by T cell activation as measured by thymidine incorporation in vitro, or by antibody formation by B cells in the *in vitro* induction of primary and secondary immune responses as measured by plaque formation.

Major Findings: A murine T lymphocyte proliferation assay which used antigen primed lymph node T cells, was antigen specific, and required exogenous accessory cells was used to characterize the accessory cells which supported proliferation. These cells were Thy 1.2 negative, radioresistant, glass-adherent, and were only functional if alive. The accessory cell function of spleen adherent cells was proportional to the length of time such cells were incubated with antigen and very small numbers of such cells provided accessory cell function. Cytotoxic studies with subregion restricted anti-Ia antibodies and complement indicated that accessory cell function resided in a subpopulation of spleen adherent cells which bore the *I-A* and *I-E* or *C* subregion antigens. The function of such cells was not related to a selective ability (vs. other spleen adherent cells) to take up antigen. These data indicate that antigen specific stimulation of T lymphocyte proliferation requires at least one specific subpopulation of spleen adherent cells which can be phenotypically identified by its expression of Ia antigens and are consistent with the possibility that Ia antigens may be *Ir* gene products.

The possibility was investigated that *Ir* genes regulate the function of cells other than T or B cells in the primary IgM PFC responses to the synthetic antigens TNP-(T,G)-A--L and TNP-(H,G)-A--L. The primary PFC responses of (Responder x Nonresponder)F_1 spleen cells to both antigens were abrogated by G-10 Sephadex passage and restored by the addition of spleen adherent cells. The cell type in the spleen adherent cell population active in reconstituting the responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L was a non-T, non-B, radiation resistant, glass adherent spleen cell. The abrogated responses of G-10 Sephadex passed (Responder x Nonresponder)F_1 spleen cells to each antigen were reconstituted only by spleen adherent cells from strains which were responders to that antigen. All the spleen adherent cells tested restored the non-*Ir* gene controlled response to a third antigen, TNP-KLH.

The ability of spleen adherent cells to function as accessory cells in the primary PFC responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L was shown to be controlled by autosomal dominant genes which were shown by the use of recombinant strains to be located in the K or I-A regions of the responder H-2 complex, the same region(s) of H-2 as the Ir genes controlling overall *in vitro* and *in vivo* responsiveness to these antigens.

Significance to Biomedical Research and the Program of the Institute: Since spleen adherent cells function as accessory cells in antigen presentation to T lymphocytes and phenotypically express both Ia antigens and Ir genes, an understanding of the mechanisms underlying accessory cell function in the immune response may lead to new modes of therapy for cancer and other human diseases.

Proposed Course of Project: A further characterization of the spleen adherent accessory cell will be approached by density gradient separations, by removal of phagocytic cells and by removal of Fc receptor bearing cells. Both expression of Ia antigens and ability to support antigen-specific T cell proliferation will be assessed at each step. The mechanism of Ag uptake and processing by accessory cells will be examined by internal and surface labeling of Ia in conjunction with uptake of radiolabelled antigens under Ir gene control.

Publications

Cowing, C. and Dickler, H. B.: Expression of Ia antigens on murine macrophages. In McDevitt, H. O. (Ed.): *Ia Antigens and Ir Genes*. New York, Academic Press, Inc., 1978, pp. 163-169.

Cowing, C., Schwartz, B. D. and Dickler, H. B.: Macrophage Ia antigens. I. Macrophage populations differ in their expression of Ia antigens. *J. Immunol*. 120: 378-384, 1978.

Singer, A., Cowing, C., Hathcock, K. S., Dickler, H. B. and Hodes, R. J.: Cellular and genetic control of antibody responses *in vitro*. III. Immune response gene regulation of accessory cell function of non-T, non-B spleen adherent cells. *J. Exp. Med*., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05066-02 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Antibody Production to TNP-Modified Syngeneic Lymphocytes in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	C. B. Pettinelli	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse spleen cells sensitized in vitro to TNP-modified syngeneic spleen cells were tested for the generation of antibody-forming cells (as well as for cytotoxic function) by assaying the cultured lymphocytes in a plaque-forming cell (PFC) assay using TNP-modified sheep erythrocytes (TNP-SRC) as targets. A PFC response specific for the TNP-haptenic moiety was detected when the cultured spleen cells were from C57BL/10, B10.D2, or B10.A(5R) donors, whereas no response was detected in cultured lymphocytes from B10.A or B10.A(4R) donors. In contrast, spleen cells from all of the above mouse strains generated strong cytotoxic responses against TNP-modified self cells. These results indicate that the antibody response against the TNP hapten coupled to syngeneic cells is controlled by genetic factors mapping within the H-2 complex (possibly immune response genes) which do not affect T-cell-mediated cytotoxicity against TNP-self structures, and suggests that these antibodies and T-cell receptors recognize different antigenic specificities.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The purpose of this project was to investigate whether mouse lymphocytes can be sensitized in vitro against TNP-modified syngeneic cells to generate TNP-specific antibody-producing PFC. If PFC could be generated, it was the purpose of this research to compare in different mouse strains the PFC and T-cell mediated cytotoxic responses against TNP-self and to determine what self components are recognized as carrier for PFC generation.

Methods Employed: Mouse spleen cells from different inbred mouse strains were sensitized in culture against TNP-modified syngeneic cells. The cells harvested from the cultures were assayed for PFC on TNP-modified SRC, and for cytotoxic activity on ^{51}Cr-labelled TNP-modified syngeneic lymphoid target cells.

Major Findings: TNP-specific PFC were generated by spleen cells from C57BL/10 and B10.A(5R) donors, but not from B10.A and B10.A(4R) donors. Cytotoxic responses against TNP-self were generated in the same cultures from all of the above strains. These results indicate that genetic factors mapping in the K and/or I-A regions of the H-2 complex control TNP-specific antibody-forming cell production.

Significance to Biomedical Research and the Program of the Institute: The finding that hapten-specific antibody can be produced when hapten is coupled to self carrier may have significance in autoimmunity. The observation that the ability to produce hapten-specific antibody on self carrier is controlled by H-2 raises the possibility that autoimmune phenomena may be linked to the MHC. This model also permits an analysis of the mechanisms leading to the production of cytotoxic effector cells and antibody-forming cells in the same cultures.

Proposed Course of Project: This project is being terminated as a separate entity, and is now included in Project No. Z01-CB-05038-06-I.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05067-03 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to TNP-Modified Autologous Lymphocytes in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	J. S. Shaw	Clinical Associate	I NCI
	D. L. Nelson	Senior Investigator	M NCI
	W. Lewis	Senior Investigator	D NCI
	A. Dattner	Clinical Associate	D NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cultures of peripheral blood leukocytes from a series of human donors were stimulated in vitro against TNP-modified autologous leukocytes. Proliferative and cytotoxic T cells were obtained after primary, secondary and teritary in vitro stimulation. The cytotoxic effectors exhibited TNP-dependent activity against modified murine lymphocytes. Blocking studies demonstrated inhibition of cyto toxic activity by addition of TNP-modified autologous targets, but not by TNP-lysine or TNP-conjugated chicken red blood cells. This establishes that the T-cells do not recognize TNP exclusively but TNP in association with human-specific self surface determinants. These determinants have been classified into three groups based on studies of direct lysis and cold target cell inhibition using panels of effectors from family members and selected unrelated donors. One class of determinants was polymorphic and associated with serologically defined HLA-A and -B locus antigens. A second class of determinants was not strongly associated with these antigens but was polymorphic and HLA-linked. A third class of "public" determinants existed for which there was little if any polymorphism among the donors studied. These results from the study of human TNP-dependent cell mediayed cytotoxicity suggest that T cells recognize a variety of self-determinants in association with TNP.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The purpose of this project was to determine whether human lymphocytes can recognize and respond to TNP-modified autologous leukocytes in ways similar to that observed using mouse lymphocytes, and if human modified self reactivity can be detected, to determine if self HL-A coded products are involved in the recognition, to map such products within the HL-A complex, and to determine whether immune response genes are involved in such T-cell responses.

Methods Employed: Human peripheral blood leukocytes were obtained from normal adult volunteers, separated on a Ficoll-Hypaque gradient, and sensitized in vitro in primary and secondary cultures against TNP-modified and unmodified autologous and allogeneic (unrelated) leukocytes. Proliferation of the responding lymphocytes was assessed by pulse-labelling with ^{3}H-thymidine and by ^{51}Cr-release assays at various times after primary, secondary, and tertiary sensitization. Cell separations were performed by: (a) depletion of effector cells of Fc-receptor-bearing cells by incubation on antigen-antibody coated plastic surfaces; (b) sequential fractionation on Sephadex G-200 anti-human F(ab')$_2$ columns followed by incubation on antigen-antibody coated plastic; and (c) rosette formation with sheep erythrocytes.

Major Findings: Peripheral blood leukocytes from all normal human donors (30/30) studied generated trinitrophenyl (TNP)-dependent cytotoxic activity following *in vitro* sensitization with TNP-modified autologous cells. Weak cytotoxic activity was generated during primary culture, increased approximately 100-fold during secondary stimulation, and another 5-fold during tertiary stimulation. The cytotoxic activity was shown to be TNP-dependent at both the sensitization and the lytic phases. Effectors lysed not only TNP-modified autologous cells but all TNP-modified human leukocyte targets tested, including those which shared no serologically-defined HLA-A; B or -C locus determinants with the specific targets. This crossreactivity appeared to be species specific, since it was not observed when human effectors were tested on TNP-modified mouse targets. The TNP-dependent cytotoxic activity on autologous and unrelated cells was shown to be mediated by T-cells without requirement for Fc-bearing cells; cytotoxic activity was recovered in subpopulations of cells positively selected by sheep erythrocyte rosetting techniques and in subpopulations negatively selected by depletion of phagocytic cells, sIg-positive lymphocytes, and Fc-receptor positive lymphocytes. Unlike ADCC mediated by anti-TNP antibody, the cytotoxic activity was inhibited by TNP-modified autologous cells, but not by TNP-lysine, or TNP-conjugated chicken red blood cells. Specificity of the classes of self determinants recognized in association with TNP was investigated by direct lysis and cold target cell competition. Interpretation of the results of direct lysis experiments was facilitated by the use of interaction analysis. Studies of effector populations from six siblings demonstrated that on the average approximately 40% of the cytotoxic activity was specific for polymorphic determinants of which most or all were HLA-linked. Studies of effectors from six unrelated donors, selectively matched for HLA-A and -B locus determinants, demonstrated that some of the cytotoxic activity was directed against HLA-A and/or -B locus-associated polymorphic determinants, but that the serologically-defined HLA-A and -B locus antigens were not the only polymorphic determinants recognized. Studies by cold target cell competition demonstrated that some of the cytotoxic activity was directed against TNP in association with relatively nonpolymorphic antigens. Thus, there was evidence for considerable diversity of the classes of self determinants which human T-cells can recognize in association with TNP. Furthermore, the possibility of immune response gene control of the cytotoxic response to these determinants was suggested by differences between donors in their response to TNP-modified self determinants.

Significance to Biomedical Research and the Program of the Institute: This project is of fundamental immunological importance in that it provides one of the first known examples of cell-mediated immunity involving self-recognition by human lymphocytes, and it can be used to test a number of major histocompatibility-linked immune phenomena in man which are known to occur in mice. It may also be useful for HL-A typing of human lymphocytes for possible new immune functions and for investigating immune response genes in man without having to immunize human subjects. The finding that certain polymorphic determinants expressed on the lymphocytes of all humans tested (but not on mouse lymphocytes) raises the possibility that these represent heretofore unidentified structures which are important components of associative recognition involving self and foreign antigens. Preliminary studies using lymphocytes from DNCB sensitized donors restimulated in culture with DNP-modified syngeneic cells indicated that effectors were generated which lysed DNP- but not TNP-modified syngeneic targets. This response exhibited no crossreactive lysis on modified HLA-unrelated targets, in contrast to the TNP system.

Proposed Course of Project: Additional studies will be carried out using lymphocytes from unrelated HL-A matched and unmatched individuals, and between members of families including those known to be recombinants within the HLA complex in order to further characterize the cytotoxic response to TNP-modified self. Attempts will be made to clone subpopulations of human lymphocytes to obtain sets of effector cells which will be more restricted with respect to the self structures they recognize. Other approaches of sensitization will be used in an attempt to exclude stimulation of the clones recognizing the polymorphic self determinants. The response potential of lymphocytes from individuals will be compared for possible differences that could be attributed to immune response genes and linkage for subregions of HLA will be tested.

Publications

Shearer, G. M., Schmitt-Verhulst, A.-M., Shaw, S., Pettinelli, C., Henkart, P. and Rehn, T. G.: The role of MHC genes in T-cell mediated responses to syngeneic modified cells. In Sercarz, E., Herzenberg, L. A., and Fox, C. F. (Eds.): Immune System: Genetics and Regulation. New York, Academic Press, 1977, pp. 577-592.

Shaw, S., Nelson, D. L. and Shearer, G. M.: Human cytotoxic response *in vitro* to trinitrophenyl-modified autologous cells. I. T-cell recognition of TNP in association with widely shared antigens. *J. Immunol.*, in press.

Shaw, S. and Shearer, G. M.: Human cytotoxic response *in vitro* to trinitrophenyl-modified autologous cells. II. Diversity of self determinants recognized in association with TNP. *J. Immunol.*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05068-02 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunology of Model Membrane Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. A. Henkart	Senior Investigator	I NCI
OTHER:	C. K. Neels	Microbiologist	I NCI
	G. M. Shearer	Senior Investigator	I NCI
	A.-M. Schmitt-Verhulst	Expert	I NCI

COOPERATING UNITS (if any)
D. Wolf, W. Webb, Department of Applied Physics, Cornell Univ., W. Strober, Metabolism Branch, DCBD, NCI, NIH.

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Synthetic amphiphilic macromolecules composed of stearic acid covalently linked to fluroescent dextran derivatives were synthesized as models for membrane proteins. These water-soluble molecules adsorb to all artificial and cell membranes tested. In the past year we have principally studied the lateral diffusion and qualitative behavior of these molecules on the surface of fibroblasts. The lateral diffusion constant is $2x10^{-10}$ cm^2/sec., a rate typical of membrane proteins. Addition of antibody to these dextrans causes patching and capping of these molecules. The lateral diffusion of these molecules is reduced by antibody crosslinking at lower concentrations than gives rise to visible patching. In the case of very flat fibroblasts these adsorbed membrane molecules form patches in rows as has been observed for natural membrane antigens. On lymphocytes, the study of capping has been extended to thymocytes which show quantitative differences from splenocytes with respect to kinetics and dose dependence, but show a similar drug inhibition profile. In addition the technology developed was used to allow the formation of IgA coated erythrocytes which have successfully detected IgA specific Fc receptors on mouse lymphocytes.

Project Description

Objectives: To test the properties of synthetic membrane antigens in cell membranes and compare these with natural membrane antigens in the same cells. In particular, a molecular explanation of the capping phenomenon which occurs upon crosslinking membrane antigens is sought.

Methods Employed: Dextran and other polysaccharide derivatives containing covalently linked TNP, rhodamine and stearate were synthesized. Saline solutions of these compounds (10-1000 μg/ml) were incubated with cells followed by washing. The adsorbed antigen was then followed by using the fluorescence microscope with direct visualization or by staining with rhodamine labeled anti-TNP. Quantitative measurements of lateral diffusion were made using fluorescence photobleaching recovery. IgA coated ox erythrocytes were made by adsorbing stearoylated dextrans and arabinogalactans to the erythrocytes and then incubating with QUPC 52 and SAPC 10, IgA myeloma proteins which bind the respective polysaccharide. These red cells were incubated with lymphocytes and found to form rosettes by standard techniques.

Major Findings: Stearylated dextrans adsorbed on the membrane of fibroblasts undergo patching and capping when crosslinked by antibody. These processes are remarkably similar to those seen with natural fibroblast antigens. On flattened cells patches form in rows, very similar to those shown to correlate with intracellular myosin by other groups. A quantitative study of lateral diffusion of these molecules showed that they move at the same rate as the less restricted membrane proteins previously studied, i.e., with a lateral diffusion constant of 2×10^{-10} cm^2/sec. When crosslinked by low concentrations of antibody the lateral diffusion is greatly retarded, even under conditions where no patches are visible. A continuation of the study of these molecules on lymphocyte surfaces showed that thymocytes adsorbed these molecules from solution more readily than splenocytes, showed an optimum surface concentration for capping, and capped the adsorbed species more slowly than splenocytes. The drug inhibition of the capping on these cells, like stearoylated dextrans on spleen cells, was very similar to drug inhibition of capping of natural markers such as sIg. In the IgA Fc receptor collaborative study with Warren Strober, Metabolism Branch, 4-6% of normal mouse spleen cells were found to form IgA rosettes, implying that this class of antibody has its own Fc recptor.

Significance to Biomedical Research and the Program of the Institute: This project is aimed at elucidating basic aspects of biological membranes, especially the behavior of antigens on cell surfaces. Such antigens play a vital role in graft and tumor rejection. The studies undertaken have revealed that simple model membrane molecules passively inserted into membranes show many of the properties of natural membrane antigens, including capping.

Proposed Course of the Project: We are still seeking methods of modifying proteins so that they become amphipathic and will insert into cell membranes. Such reagents would have a number of important practical applications.

Publications

Wolf, D. E., Schlessinger, J., Elson, E. L., Webb, W. W., Blumenthal, R., and Henkart, P.: Diffusion and patching of macromolecules on planar lipid bilayer membranes. Biochemistry 16, 3476-3483 (1977).

Henkart, P. A., Schmitt-Verhulst, A.-M., and Shearer, G. M.: Specificity of cytotoxic effector cells directed against trinitrobenzene sulfonate-modified syngeneic cells: Failure to recognize cell-surface-bound TNP-dextran. J. Exp. Med. 146:1068-1078, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05069-02 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Expression of Ia Antigens on Functional Cell Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. B. Ahmann	Clinical Associate	I NCI
OTHER:	R. J. Hodes	Head, Immunotherapy Section	I NCI
	D. H. Sachs	Head, Transplantation Biology Section	I NCI
	A. Singer	Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been demonstrated that pre-treatment of mouse lymphoid cells with anti-Ia (I-region-associated) sera and complement (C) abrogates the T-cell proliferative response to Con A and lentil lectin, but not PHA. Reconstitution experiments have shown that a glass adherent, non-T, non-B, 1000R resistant accessory cell is the target in the abrogation of the response to Con A by anti-Ia and C. Genetic studies have shown that the Ia determinants expressed on these accessory cells are encoded by at least two different subregions of the I-locus. These determinants appear to be expressed on the same cell. Treatment with anti-Ia sera and C has also been shown to prevent the generation of CML (cell-mediated lympholysis) suppressor cells induced by 5-day culturing of normal spleen cells. These Ia+ accessory cells have also been shown to be essential for the in vitro generation of Ir gene controlled as well as non-Ir gene controlled T-cell-dependent IgM responses to soluble TNP-conjugated protein antigens.

Project Description

Objectives: Ia antigens have been associated with many immunologically-related phenomena. Using defined Ia alloantisera, it has been possible to map the genes to subregions that encode particular Ia determinants. The objective of this study is to use these antisera to identify markers for cells involved in specific immunological function (e.g. Con A reactive cells, accessory cells, suppressor cells, helper cells, etc.) and from this hopefully to clarify the mechanisms of action of these cells, as well as the role of Ia antigens in these responses. Three response systems chosen for study are (1) the proliferative response to Con A, (2) the *in vitro* generation of T-cell dependent IgM responses to soluble TNP-conjugated protein antigens, and (3) the generation of CML (cell mediated lympholysis) suppressor cells induced by 5-day culturing on normal spleen cells.

Methods Employed: In each of the three response systems being studied, the cellular requirements are being analyzed, using expression of Ia antigens on cellular subpopulations as a probe for this analysis. (1) For analysis of Con A response, responding cells are treated with anti-Ia sera and C, the resulting cells cultured *in vitro* with mitogens, and the proliferative response determined. Cell separation and reconstitution techniques are being employed to determine which cell population (or populations) is eliminated by the anti-Ia sera and C treatment. (2) In the *in vitro* primary IgM response to soluble antigens, requirements have been established for T cells, B cells, and accessory cells. Separated subpopulations are treated with anti-Ia and C, and assessed for activity in *Ir* gene controlled antibody responses to TNP-(T,G)-A--L and in non-*Ir* controlled responses to TNP-KLH. (3) The cellular requirements for in vitro generation of T cell suppressors of CML are being assessed by cell fractionation and anti-Ia treatment of suppressor precursors and of differentiated suppressor cells.

Major Findings: (1) It was shown that pretreatment of murine lymphoid cells with anti-Ia and C abrogated their ability to respond to Con A. Reconstitution experiments have shown that glass adherent spleen cells treated with a T cell-specific rabbit anti-mouse brain serum and C and irradiated with 1000 R (spleen adherent cells, SAC) were able to restore responsiveness to Con A whereas T cell-enriched and B cell-enriched populations did not restore Con A responsiveness. (2) In a second assay system, T cell-dependent IgM responses of normal spleen cells to soluble TNP-conjugated protein antigens were abrogated by Sephadex G-10 passage. Responses in this system were similarly reconstituted by the addition of Ia$^+$ SAC as accessory cells. Using anti-Ia sera specific for certain *I*-subregions it was shown that these SAC expressed Ia determinants encoded by genes in *I-A* and *I-E/C*. In addition, these determinants appear to be expressed on the same cell. (3) Treatment of lymphoid cells with anti-Ia sera and complement abolished the generation of CML suppressor cells by 5-day culturing of normal spleen cells, but did not prevent them from responding in CML or MLR. Passage of spleen cells through Sephadex G-10 also abolished the generation of CML suppressor cells by 5-day culturing of normal spleen cells. Thus Ia$^+$ SAC appear to play a significant role in 3 murine response systems.

Significance to Biomedical Research and the Program of the Institute: Ir gene products play a significant role in regulation of immune responses. A basic understanding of the expression, and perhaps the functional role of one class of I region gene products, Ia antigens, in mitogen responses, in antibody production, and in T cell-mediated suppression of CML should provide insight into mechanisms of control of these immune responses. Such insight would be relevant to evaluation of the host response to allograft and tumor challenge.

Proposed Course of Project: Studies are in progress to further define the properties of the Ia^+ accessory cell and its mechanism of action. Genetic studies will also be performed to determine which gene(s) control expression of the Ia determinants necessary for the generation of a suppressor cell in CML. Work will also be done to determine the presence of soluble Ia factors in serum which appear to be important in the Con A response of purified T cells.

Publications

Ahmann, G. B., Sachs, D. H., and Hodes, R. J.: Genetic analysis of Ia determinants expressed on Con A reactive cells. In Sercarz, E. E., Herzenberg, L. A., and Fox, D. F. (Eds.) Immune System: Genetics and Regulation, New York, Academic Press, 1977, pp 249-256.

Ahmann, G. B., Sachs, D. H., and Hodes, R. J.: Ia determinants on accessory cells essential for the response of murine lymphoid cells to Con A. Fed. Proc. 1978 (in press).

Ahmann, G. B., Sachs, D. H., and Hodes, R. J.: Genetic analysis of determinants on Con A reactive cells. J. Immunol., 1978 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CB-05075-01 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Liposome-Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. D. Leserman	Clinical Associate	I	NCI
OTHER:	J. N. Weinstein	Senior Investigator	LTB	NCI
	R. P. Blumenthal	Visiting Scientist	LTB	NCI
	W. D. Terry	Associate Director for Immunology	I	NCI
	S. O. Sharrow	Chemist	I	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

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NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Liposomes in which fluorescent compounds have been included by sonication have been incubated with myeloma tumor cells, phagocytic cells, and various other lymphoid cell populations. Use of specific antigens in the liposome membrane has permitted quantification of surface immunoglobulin on MOPC 315 murine myeloma cells by fluorescence-microscopy, by fluorometry and by use of the fluorescence activated cell sorter. Inclusion of cytotoxic drugs within liposomes has shed light on the relationship between surface binding and drug-delivery to the cytoplasm. Current data suggest enhanced cytotoxic effect when drugs are liposome-bound, in cell populations which can incorporate these liposomes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To examine the interaction of several types of normal and malignant cells with lipid vesicles containing fluorescent markers or chemotherapeutic agents. To determine the relationship between lipid surface antigens and the binding of liposomes to cells. To optimize binding and transfer of liposomal contents to tumor cells in attempts to promote specific delivery of drugs to tumor cells.

Methods Employed: Single-bilayer vesicles are made from synthetic phospholipid and cholesterol. In some studies hapten-derivatized phospholipids have been included within the vesicle membrane. The fluorescent compound carboxyfluorescein has been included within the vesicles by sonication, either alone or in conjunction with various drugs. Interactions of cells with vesicles has been monitored by fluorescence microscopy, by fluorometry and by use of the fluorescence activated cell sorter. Drug-delivery to tumor cells has been monitored by counts of surviving cells and by uptake of radionuclides.

Major Findings: Surface immunoglobulin of MOPC 315 murine myeloma cells has been quantitated by incubation with fluorescent liposomes bearing the DNP-hapten, for which the immunoglobulin has affinity. Encapsulation of methotrexate in vesicles permits effective release of the drug into phagocytic tumor cells in vitro; preliminary results suggest that vesicle-bound drug has a greater inhibitory effect on these tumor cells than the equivalent concentration of free drug.

Significance to Biomedical Research and the Program of the Institute: Use of fluorescent vesicles bearing surface antigens has shown promise as a new marker for surface immunoglobulin. Studies on the interaction between liposomes containing drugs and tumor cells have a direct bearing on attempts to target therapy to specific tumors.

Proposed Course of Project: Methods to couple additional markers such as protein antigens to liposomes will be evaluated. Alterations in phospholipid will be made, in order to maximize release of vesicle bound drugs to tumor cells. Interaction between vesicle and phagocytic cells will be monitored by fluorescent measurement and by the functional perturbation of liposomes containing drugs.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05076-01 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Role of the H-2L Locus Antigens in T-Cell Mediated Cytotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. B. Levy	Postdoctoral Fellow	I NCI
OTHER:	T. H. Hansen	Postdoctoral Fellow	I NCI
	W. E. Biddison	Guest Worker	I NCI
	G. M. Shearer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

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NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Balb/c H-2^{db} spleen cells generated a strong primary in vitro CML response against Balb/c (H-2^{d}) wild type spleen cells. The effector cells generated were specific for L antigens. In an allogeneic CML response between two non-mutant strains directed against H-2D region antigens, two clones of cytotoxic effector cells could be identified, one with specificity for D antigens, and the second with specificity directed against L antigens. When syngeneic cytotoxic effector cells were generated against TNP modified H-2D antigens, no clones with specificity for L-TNP modified antigens could be detected. Therefore, although L alloantigens are capable of inducing allogeneic cytotoxic effector cells, these antigens may not be capable of elliciting syngeneic effector cells specific for TNP modified antigens.

Project Description

Objectives: The objective of this project was to determine the ability of L-locus products of the H-2D region to stimulate the generation of allogeneic cytotoxic effector cells. Additionally, the ability of chemically (TNBS) and virally (Hong Kong strain of influenza) treated H-2L antigens to stimulate syngeneic H-2-restricted cytotoxic effector cells is also under investigation.

Methods Employed: Spleen cells from Balb/c $H\text{-}2^{db}$ mutant mice (a lost type mutant in which L-locus products are antigenically undetectable) and other mouse strains were cultured for 5 days against either spleen cells, or TNBS treated spleen cells. The effector cells from these cultures were harvested and assayed on ^{51}Cr labelled target cells. The use of non-radioactively labelled ("cold") spleen cells (i.e. cold target inhibition) and specific anti-H-2 antisera (i.e. antiserum blocking) were frequently employed.

Major Findings: Balb/c $H\text{-}2^{db}$ spleen cells generated cytotoxic effector cells after stimulation with Balb/c ($H\text{-}2^{d}$) wild type cells. Antiserum blocking experiments showed that these effector cells were specific for the L antigen(s). When two non-mutant strains were used to generated cytotoxic cells against H-2D region differences, it was found (by cold target inhibition and antibody blocking techniques) that at least two clones of effectors could be identified, one clone specific for L antigens, and a second clone with specificity for D antigens. When autologous TNP-modified spleen cells were used to stimulate responding spleen cells, the resultant H-2D-TNP specific effectors were examined for the presence of L-TNP specific killers. Using cold target inhibition and antibody blocking methods, effector cells generated against putatatively TNPmodified H-2L products were not detected. Therefore, although the L alloantigens are capable of inducing allogeneic cytotoxic effector cells, they may not be capable of inducing syngeneic effector cells specific for TNP modified antigens.

Significance to Biomedical Research and the Program of the Institute: This work extends the understanding of genetic loci and their products which map in the major histocompatibility complex (MHC) and examines their immunological relevance for T-lymphocyte recognition. It also provides information concerning the immunological implications involved in mutations within the MHC.

Proposed Course of Project: The ability of L-locus products to stimulate and serve as target antigens for cytotoxic effector cells will be further examined in order to determine whether these antigens are analogous to association antigens (H-Y, minor histocompatibility, etc.), or similar to H-2K and H-2D products. The significance of L-locus products in the generation of cytotoxic effector cells against influenza infected autologous cells, and the role of these antigens in the stimulation of lymphocyte proliferation (MLR) will also be examined.

Publications

Hansen, T.H., Levy, R.B.: Alloantigens determined by a second D region locus ellicit a strong in vitro cytotoxic response. J. Immunol. in press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05077-01 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

"Autosensitization" In Vitro Detected by Cell-Mediated Cytotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	R. B. Levy	Postdoctoral Fellow	I NCI
	J. Kim	Postdoctoral Fellow	I NIAID
	R. Asofsky	Senior Investigator	I NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.8	OTHER: 0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

T-cell-mediated cytotoxicity has been generated by culturing immune spleen cells from untreated donors without the addition of any known cellular antigens. The effectors generated preferentially lysed H-2 matched targets, but also lysed to some extent allogeneic target cells. High ($H\text{-}2^d$) and low ($H\text{-}2^{k,b}$) responder strains were identified, and strong responsiveness was a dominant characteristic. Specificity studies suggested that the effector cells were sensitized against components contained in fetal calf serum, but not in horse serum. Enhanced responses could be obtained by preinjecting mice with irradiated syngeneic cells which raised the possibility that the response is dependent on natural killer cells.

Project Description

Objectives: The objective of this project was to investigate the requirements for the generation of "autosensitized" effector cells. Furthermore, the antigens involved in the stimulation of these effector cells, as well as the antigens involved in the lytic phase were examined.

Methods Employed: Spleen cells from various mouse strains were cultured in vitro for 5 days, and assayed on a number of ^{51}Cr-labelled H-2 matched or unmatched target cells. Non-radioactively labelled (°cold°) tumor cells were used to inhibit the cytotoxic effector cells.

Major Findings: Cytotoxic effector cells were detected after mouse spleen cells were cultured in vitro for 5 days without the addition of non-self (H-2) or modified stimulator cells. The effector cells were shown to be T-lymphocytes. Direct cytotoxic lysis indicated that H-2 matched target cells were lysed to a greater extent than H-2 unmatched targets. Cold target inhibition studies confirmed these results and suggested that at least two clones of cytotoxic effectors were present: one clone inhibitable with H-2 matched cells only, another clone inhibitable with either H-2 matched or H-2 unmatched cells. Of several strains examined, spleen cells from $H\text{-}2^d$ haplotype strains generated signficantly greater (high responder) levels of cytotoxicity than $H\text{-}2^b$ and $h\text{-}2^k$ (low responder) spleen cells. High responsiveness appeared to be dominant, since cells from F_1 hybrids of high and low responders generated strong responses.

The generation of these effector cells appeared to depend on the serum source used to supplement the sensitization medium, since only spleen cells cultured in fetal calf but not horse serum generated this lytic activity. The incubation of target cells in various serum sources supported these conclusions. Target cells assayed in fetal calf serum were significantly more susceptible to "autosensitized" effector cells than the same target assayed in a different serum source (horse, minipig). The cytotoxic potential of splenic lymphocytes could be enhanced by injecting prospective responding cell donors with irradiated (1500 R) syngeneic spleen cells 5-12 days before culture.

Significance to Biomedical Research and the Program of the Institute: This study provides an example of major histocompatibility restricted cytotoxicity in which the modifying agent (some component of fetal calf serum) does not require covalent linkage to or infection of the cells. It permits a study of fetal serum proteins with respect to their ability to interact with tumor cells and the immunogenicity resulting therefrom. This work also has implications concerning the nature of immune recognition of syngeneic tumor cells maintained in fetal calf serum supplemented media.

Proposed Course of Project: Various serum sources will be examined for their ability to support the generation of "autosensitized" effector cells. The significance of various serum components (ex. fetal proteins) in the induction of this response will be investigated, since the difference in the susceptibility of various tumor cells to autosensitized effectors, as well as the high and low responsiveness of various H-2 strains could be a result of the ability of each cell type to bind a critical serum component. The genetic aspects of the high and low responder strains will be further studied. The mechanism of enhancement of the response by pre-injecting irradiated cells will be investigated, and the possible role of a natural killer-like cell will be examined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05078-01 I

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Influenza-Infected Autologous Lymphocytes in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	W. E. Biddison	Guest Worker	I NCI
	J. S. Shaw	Clinical Associate	I NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Conditions have been developed for: (a) in vitro stimulation of influenza virus-immune cytotoxic effector cells in human peripheral blood leukocyte (PBL) populations; and (b) measurement of effector cell activity on virus-infected cryopreserved human PBL target cells. Effector cells generated by in vitro stimulation with one type A influenza virus A/HKx31 (H3N2) were cytotoxic for autologous target cells infected with both A/HKx31 and a serologically distinct type A influenza virus PR8 A/Puerto Rico/8/34 (H0N1), but did not kill cells infected with a type B influenze virus (B/Hong Kong). Conversely, effector cells generated against B/Hong Kong lysed B/Hong Kong, but not A/HKx31 or PR8 virus-infected autologous cells. These results indicate that the human T cell response to influenza viruses may be type-specific. They also appear to be restricted to recognize self components.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project are to: (a) define the components of the human major histocompatibility complex (HLA) which are recognized in association with viral antigens by cytotoxic T cells; (b) analyze the virus specificity of human influenza-immune cytotoxic T cells; (c) determine if there is any "Ir-like" genetic control of human T cell response potential to influenza viruses; and (d) characterize the human cell populations required to generate virus-immune cytotoxic T cells.

Methods Employed: Human PBL were obtained from normal adult volunteers, separated on a Ficoll-Hypaque gradient, and sensitized *in vitro* to live influenza viruses. Cytotoxic activity was measured by ^{51}Cr release from cryopreserved peripheral blood cells which were thawed, stimulated with PHA, and infected with influenza virus.

Major Findings: At this point, four major findings have been made: (a) human influenza-immune cytotoxic effector cells can be induced *in vitro*; (b) suitable virus-infected target cells can be prepared from cryo-preserved cells obtained from any given individual; (c) influenza-immune effector cell populations appear to recognize type-specific viral components expressed on the surface of virus-infected PHA blasts; and (d) the cytotoxic responses appear to be very self restricted.

Significance to Biomedical Research and the Program of the Institute: This project is of fundamental immunological importance, since: (a) it provides one of the first known examples of cell-mediated immunity which involves self-recognition by human lymphocytes, and (b) it involves a virus which is of significance in human disease. Furthermore, this system can be used to test a number of major histocompatibility-linked immune phenomena in man which are known to occur in mice.

Proposed Course of Project: Studies will be performed using lymphocyte populations from unrelated HLA matched and unmatched individuals, and from members of families including those known to be recombinants within the HLA complex in order to define the components of the HLA complex which are recognized in association with viral antigens. Individuals will also be assessed for differential T-cell response potential and, it such differences can be identified, studies will be performed to determine whether they can be attributed to HLA-linked traits. Precursor populations and cytotoxic effector cells will be characterized based on E-rosette formation as well as presence or absence of Fc receptors and surface immunoglobulin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05081-01 I

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Ontogeny of Immune Responsiveness in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. I. Nadler	Clinical Associate	I	NCI
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of many mammalian species to respond to in vivo challenges with complex antigens varies in a time dependent manner during embryonic and neonatal life. In utero and at birth immunologic competence is incomplete but the precise nature of the defect or defects is poorly understood. Studies are under way to elucidate the nature of immunologic unreactivity in neonatal mice utilizing a system of in vitro primary antibody response. It is of interest to determine how environmental exposure to antigens and the mammalian genome interact in controlling phenotypic expression of immune reactivity throughout development. Further studies may shed light on the time course of development of self tolerance.

Project Description

Objectives: Investigation is under way concerning the maturation of the immune response in mice to soluble hapten-protein conjugates. Inability to respond to various antigens has been noted in many species during embryonic development and in the neonatal period. Immunologic competence to distinct antigenic determinants is achieved in an orderly progression in animals of mammalian species thus far studied. Studies in progress have demonstrated that prior to seven to ten days after birth splenic lymphocytes from BALB/c mice fail to respond in vitro to soluble KLH-TNP as measured by a plaque-forming cell assay of primary antibody response. The ability to respond increases over the period from 1 week after birth until maximal responses are seen in animals 6-8 weeks of age. The nature of neonatal unresponsiveness in vivo and in vitro is unclear. Postulated mechanisms for this failure to response to antigens include: (1) The presence of non-specific and/or antigen specific suppression (cell or "factor" mediated); (2) inability of accessory cells to "process antigen"; (3) absence or functional immaturity of antigen specific precursors at various stages of maturation despite presence of pluripotent stem cells; or (4) immaturity of cell-cell cooperative mechanisms. The aim of these studies is to elucidate which of these mechanisms is responsible for neonatal immunlogic incompetence and how adult immunologic reactivity is achieved.

Methods Employed: The major experimental system employed involves in vitro primary antibody responses as measured in a plaque-forming cell assay. Cell separation procedures include nylon non-adherence to obtain populations enriched in T cells; RαMB and complement treatment to obtain B cell preparations and glass adherence for macrophage or "adherent cell" populations.

Significance to Biomedical Research and the Program of the Institute: It is known that immune responsiveness in neonatal and senescent animals is lower than in adult animals of that species. Information concerning the mechanisms of neonatal immune deficiency is incomplete and investigations in this area will be applicable to the process of differentiation and to possible means of modulation of responsiveness. It is also known that the incidence of malignant neoplasms is greatest at the extremes of life and it has been postulated that defects in "immunologic surveillance" may play a role in this occurrence.

Proposed Course of Project: Studies will pursue the cellular causes of the inability of neonatal animals to respond to soluble antigens. The mechanism whereby neonatal spleen cells inhibits the immune response of syngeneic adult spleen cells is also being investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05079-01 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Study of Protein Nucleic Acid Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shifrin	Chemist	I	NCI
Other:	C. L. Parrott	Chemist	I	NCI
	U. Weiss	Chemist	LCP	NCI
	T. Karpetsky	Chemist	BCRC	NCI
	C. C. Levy	Chemist	BCRC	NCI
	I. M. Chaiken	Chemist	LCB	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	1.4	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project was to study the nature of the forces through which proteins and nucleic acids recognize one another. The protein which was examined was pancreatic ribonuclease-A, ribonuclease-S, S-protein, S-Peptide and various chemical modifications. The model for the nucleic acid was polyadenylic acid, oligomers of adenylic acid and various derivatives. Interactions were measured by analytical and sucrose gradient ultracentrifugation as well as by gel chromatography, ultraviolet absorption spectroscopy and fluorescence.

Project Description

Objectives: Polyadenylic acid, whether added to RNA or covalently attached to the 3'-terminus as found with many mRNA species, prevents the hydrolytic action of ribonuclease. In the presence of spermine or Mn^{+2} the enzyme has full hydrolytic activity and the nucleic acid is degraded. The purpose of the study was to determine the nature of the functional groups of RNase which interact with poly(A) as well as the functional groups of the purine which participate in the interaction.

Methods Employed: Chemical modification of protein functional groups, characterization of the modified protein, isoelectric focusing, affinity chromatography, sucrose density gradient ultracentrifugation, analytical ultracentrifugation, gel chromatography, ultraviolet absorption spectroscopy, fluorescence, autoradiography.

Major Findings: A. Native ribonuclease-A slowly hydrolyzes poly(A). The polyadenylic acid is protected by spermidine or $MnCl_2$ against the hydrolytic activity of the enzyme. While treatment of the enzyme with iodoacetic acid inhibits catalytic activity towards RNA or cCMP, carboxymethyl-RNase has full activity towards poly(A).

Modification of tyrosyl residues by either iodination or nitration does not affect hydrolytic activity towards RNA or poly(A) although treatment of the enzyme with acetylimidazole which has been reported to be selective for tyrosyl residues does inhibit the activity against poly(A) with no change in activity towards RNA or cCMP.

Treatment of RNase-A with increasing amounts of succinic anhydride causes a very small decrease in activity towards cCMP and RNA but produces a complete loss of activity towards polyadenylic acid. The degree of succinylation of the protein was followed by isoelectric focusing as well as by using ^{14}C-labelled succinic anhydride. The results indicate that succinylation of two lysyl residues out of a total of ten can completely destroy the hydrolytic activity towards poly(A). While the hydrolysis of cCMP or RNA by native RNase is completely inhibited by poly(A), the polynucleotide has no effect on the hydrolysis of these two substrates using succinyl-RNase.

There are other indications that the active site of ribonuclease towards poly(A) is not the same site as that against cCMP or RNA. For example, 2'-UMP inhibits the enzymatic hydrolysis of cCMP and RNA but enhances the rate of hydrolysis of poly(A). Ribonuclease S-protein (i.e., RNase in which the first 20 amino acid residues are missing) has no activity towards RNA or cCMP but is able to hydrolyze poly(A).

Preliminary results suggest that succinyl-RNase is still able to bind poly(A) but cannot hydrolyze the polynucleotides.

Studies are currently under investigation to determine the affinity of succinyl-RNase for a typical RNase inhibitor using affinity chromatography.

Significance to Biomedical Research and the Program of the Institute: The interaction of the polyadenylic acid portion of mRNA- poly A with RNase suggests a role for the polynucleotide in control of cellular regulation. It is becoming increasingly evident that particular cellular proteins can control cell growth and transformation by binding to nucleic acids - DNA or RNA. An understanding of the forces through which the proteins and nucleic acids recognize one another for example should permit one to synthesize drugs which could interfere with the interaction - and thus interfere with transformation.

Proposed Course of the Project: We propose to purify the T-antigen from lytically infected CV-1 cells and study its interaction with the DNA isolated from tissue culture cells. If we observe that the protein (T-Ag) and nucleic acid (DNA) do interact, we hope to chemically modify the protein with a variety of reagents in an effort to determine the functional groups involved in the interaction.

Publications

Chiang, C. C., DeCamp, W. H., Curtin, D. Y., Paul, I. C., Shifrin, S. and Weiss, U.: The color dimorphism of 14-hydroxymorphinones: X-ray analysis of two different crystalline modifications. *J. Am. Chem.Soc.*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05080-01 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Study of the Nature and Biosynthesis of and Host Immune Response Against Tumor Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Chang	Visiting Associate	I	NCI
Other:	P. T. Mora	Head, Macromolecular Biology Section	I	NCI
	S. W. Luborsky	Chemist	I	NCI

COOPERATING UNITS (if any)

D. Simmons, NIAID; R. G. Martin, LMB, NIAMDD; D. M. Livingston, Sidney Farber Cancer Center, Harvard Univ., Boston; G. Jay, PO, NCI; J. S. Rhim, Microbio-Biological Associates; R. Huebner, NCI; G. Fey and W. Topp, CSHL, New York

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the nature of tumor antigens associated with cells transformed by either SV40 virus, chemicals or spontaneous induction; and the relation of these antigens with gene expression and host immune mechanisms against these antigens. Current research is carried out on: 1) Study of the relationship between SV40 T, surface and tumor specific transplantation antigens, and their relationship to SV40 A gene expression; 2) Study of the host immune response against SV40 transformed cells; 3) Study of the immunogenicity of spontaneously transformed cells and of these cells super-infected by SV40; 4) Solubilization and characterization of common rat tumor cell antigen.

Project Description

Objectives: To elucidate certain biochemical and immunological changes in cells during transformation, their molecular mechanisms of biosynthesis and the host immune response against those antigenic changes.

Methods Employed: A) Biochemical methods: Labelling glycoprotein and proteins with radioactive precursors, column chromatography, gel electrophoresis, ultracentrifugation, tryptic peptide analysis, fractionation of subcellular components, etc. B) Immunological methods: Antibody mediated cytotoxicity, complement fixation, radioimmuno-precipitation, I^{125} protein A surface antigen tests, indirect fluorescent antibody assays, ^{51}Cr cell mediate cytotoxicity, *in vivo* transplantation-rejection assays.

Major Findings: I) The relationship of SV40 induced T, surface and tumor specific transplantation antigen (TSTA) and their relationship to SV40 A gene expression. (Work with Drs. P. T. Mora, S. W. Luborsky, R. G. Martin, D. Simmons, D. M. Livingston, G. Jay, G. Fey and W. Topp.) The major findings of this work can be summarized as follows:

1) After detergent solubilization the SV40 induced T antigen, TSSA and TSTA co-purify through ammonium sulfate precipitation, DEAE cellulose, DNA cellulose and P-cellulose columns. Further purification using sucrose gradient centrifugation indicates the SV40 TSTA sediments at the same rate as the SV40 T antigen.

2) The SV40 T antigen has TSTA activity in mice and both antigens are encoded by the SV40 A gene. This conclusion is based on the fact that T antigen is coded by the SV40 A gene and the findings that: a) The anti T antigen serum with the aid of *Staph. aureus* could quantitatively precipitate the TSTA from partial purified fractions which contained both T antigen and TSTA. b) Purified T antigen which was homogeneous by SDS polyacrylamide gel electrophoresis, with mol. wt. 92,000, was highly active in providing tumor specific transplantation immunity against SV40 transformed tumor cells in mice.

3) The location of the antigenic determinants of SV40 TSTA was investigated by using various adeno-SV40 hybrid viruses. Proteins encoded by Ad_2+ND_2 (M.W. 56,000) and Ad_2+ND_1 (M.W. 28,000) contain approximately 50% and 22%, respectively, of the carboxy-terminal portion of T antigen. These proteins have TSTA activity. The protein encoded by Ad_2+ND_1dp_2 (M.W. 23K) contains only 55 (approximately 8%) carboxy-terminal amino acid residues of T antigen. It did not have TSTA activity. Thus the amino acid sequence between 8% and 22% from the carboxy-terminus was essential for expression of the antigenic determinants of SV40 TSTA.

4) The SV40 T antigen and TSTA are located predominantly in the nucleus. The immunogenicity of intracellular T antigen and TSTA might be explained by invoking the disintegration of tumor cells with consequent exposure of intracellular antigens to immunocytes. There must be TSTA existing on cell surface which is responsible for immunosensitivity, since current ideas of

immune mechanisms against tumor cells make it difficult to conceive of an intracellular antigen being responsible for in vivo cytostatic or cytotoxic effects. However, various surface antigen measurement techniques have failed to detect reaction of anti T serum with the SV40 transformed cell surface, while anti T serum does react with SV40 TSTA when it is solubilized.

A new size class of T antigen, designated as "middle T" antigen has been found. This contrasts with the "large T" antigen (M.W. 85K to 100K) and the "small t" antigen (M.W. 17K to 21K). The middle T antigen, M.W. 56K, was identified by radioimmuno-precipitation with anti T serum of ^{35}S methionine labelled SV40 transformed cell extracts. Study of various cell lines indicated the presence of this middle T antigen in SV40 transformed human, rat, hamster, rabbit cells and also in the SV40 lytically infected monkey cells. This protein is phosphorylated. Tryptic peptide analysis of this protein revealed 14 methionine labelled peptides, 4 of them being in common with those from the 92K large T antigen and 2 in common with small "t" antigen. The relationship between the biosynthesis of this 56K T antigen and the expression of the SV40 A gene was studied by using various SV40 deletion mutants and adeno-SV40 hybrids. The results suggested the possibility of new splicing mechanisms of mRNA of the SV40 A gene, and/or extensive host post-translational modification of this A gene products, e.g., glycosylation and phosphorylation. Further work is also in progress to determine whether this protein is a membrane TSTA which is responsible for in vivo immunosensitivity in mice.

II. Cellular immune response against SV40 transformed cells. (Work with P. T. Mora.) Since the nature of SV40 TSTA is strong, quantifiable, and well characterized, it offers a good test system to answer questions related to immune surveillance. The cellular immune response was reported to play a dominant role of immune surveillance against tumor cells. Therefore, studies were carried out to measure cellular response against SV40 transformed cells, with ^{51}Cr release cell mediated cytotoxicity measurements. Very high cytotoxicity against SV40 transformed cells was found by using immune peritoneal exudate (PE) cells. Low but significant killing of SV40 transformed cells was also demonstrated with immune lymphocytes from spleen cells. This cell mediated cytotoxicity was specific to SV40 transformed cells. Kinetic studies indicated the peak activity of PE cells was at the 8th day, and at the 11th day for spleen cells, after i.p. immunization. This cytotoxicity was also shown to be H-2 restricted. Work is in progress to identify the cell type responsible for the cytotoxicity. Cytotoxic lymphocytes from in vitro sensitization with SV40 transformed cells was also obtained. However, this cytotoxicity was not limited to SV40 transformed cells. This work is in progress.

III. Study of the immunogenicity of spontaneously transformed cells and those cells super-infected by SV40. (Work with P. T. Mora.) Spontaneously transformed tumor cells, unlike virus or chemically transformed cells, were reported to have no or very low immunogenicity. Our study of the tumor associated transplantation antigen (TATA) in two independently obtained spontaneously transformed cloned cell lines, T AL/N-Cl_3 and 210, and their SV40 transformed subclones indicated the presence of TATA in spontaneously transformed 210 and in T AL/N Cl_3 cells. They were demonstrated by immunizing twice the syngeneic AL/N mice with relatively large amounts ($5x10^6$ to $1x10^7$) of x-irradiated cells. Furthermore, 210 and T AL/N-Cl_3 have their own unique TATA, since they do not cross protect with each other. No significant enhancement of the immunogenicity was observed by using the SV40 super-infected derivatives of these spontaneously transformed cells. Attempts will be made to analyze the nature of the TATA of these spontaneously transformed cells.

IV. Solubilization and characterization of common rat tumor cell antigens. (Work with R. Rhim and R. J. Huebner.) Common tumor antigens were expressed in Fisher rat tumor cells transformed by DNA tumor viruses, RNA tumor viruses, chemical and spontaneous induction. The antiserum against these antigens were obtained from Fisher rats bearing syngeneic polyoma virus transformed tumor cells. These cross-reacting antigens were localized mainly intracellularly. They were solubilized by detergent extraction. Three species of antigens with M.W. 155K, 120K and 30K were found by radioimmuno-precipitation assay in all transformed Fisher rat tumor cells obtained from various laboratories. None of these antigen species were found in untransformed rat embryo cells. This finding suggests these cross-reacting antigens were coded by the host genome but derepressed (or activated) by transformation by various means.

Significance to Biomedical Research and the Program of the Institute: Studies of the nature of tumor antigens and their relationship with gene expression and host immune response are of importance in leading to the understanding of the molecular mechanisms of cell transformation and host immune mechanisms against tumor cells.

Proposed Course of the Project: Attempts will be made to further study 1) the relationship between SV40 TSTA and the cell surface, 2) control of gene expression for the newly discovered middle T antigen and its relation with transformation, 3) the host immune response against SV40 transformed cells using purified SV40 TSTA, 4) the nature of TATA of spontaneous transformed cells, and 5) the application of common rat tumor antigen for immunological prevention of tumor cells derived from different origin.

Publications

Chang, C., Anderson, J., Martin, R. G. and Mora, P. T.: Expression of tumor specific transplantation antigens in cell lines transformed by wild type or mutant SV40 viruses. J. Virol. 22: 281-288, 1977.

Chang, C., Luborsky, S. W. and Mora, P. T.: Tumor specific transplantation antigen from SV40 transformed cells binds to DNA. Nature 269: 438-440, 1977.

Mora, P. T., Chang, C., Couvillion, L., Küster, J. M. and McFarland, V. W.: Immunological selection of tumor cells which lost SV40 antigen expression. Nature 269: 36-40, 1977.

Mora, P. T., Chang, C., Khoury, G., Küster, J. M., Luborsky, S. W. and McFarland, V. W.: Antigenic expression of and the in vivo selection against the early SV40 gene. INSERM 69: 327-336, 1977.

Mora, P. T., Pancake, S. J., Luborsky, S. W., Chang, C. and Coll, J.: Pertinence of surface membrane changes in spontaneously and virally transformed cells to the balance between tumorigenicity and immune rejection. In R. E. Harmon (Ed.): Cell Surface Carbohydrate Chemistry. New York, Academic Press, 1977, pp. 83-102.

Jay, G., Jay, F. T., Chang, C., Freidman, R. M. and Levine, R. A.: Tumor specific transplantation antigen: Use of the Ad_2+ND_1 hybrid virus to identify the protein responsible for SV40 tumor rejection and its genetic origin. Proc. Natl. Acad. Sci. U.S.A., 1978, in press.

Luborsky, S. W., Chang, C., Pancake, S. and Mora, P. T.: Comparative behavior of SV40 T antigen and of tumor specific surface and transplantation antigens during partial purification. Cancer Res., 1978, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05526-10 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Spontaneously or by SV40 Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Mora	Head, Macromolecular Biology Section	I NCI
Other:	S. W. Luborsky	Chemist	I NCI
	C. Chang	Visiting Associate	I NCI
	D. Winterbourne	Visiting Fellow	I NCI
	V. W. McFarland	Chemist	I NCI

COOPERATING UNITS (if any)

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D. Simmons, LBV, NIAID
R. G. Martin, LMB, NIAMDD
D. M. Livingston, Sidney Farber Cancer Center, Boston
R. Weil, University of Geneva, Switzerland

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the cell surface membrane, how it may change in transformation to malignancy, either spontaneously, or as induced by SV40 virus. Currently research is carried out on 1) Changes in glycoconjugates, including glycoproteins and sulfated glycosoaminoglycans such as heparan sulfate, and correlation with cell growth properties in tissue culture and in vivo in the syngeneic and in the nude mice; 2) Controls of expression and methods of purification of SV40 specific T antigens, surface antigens and tumor specific transplantation antigens (TSTA); 3) Selection of phenotypic and genotypic revertant tumor cells which lose SV40; 4) Study of "spontaneous" transformation of cells in culture and of the balance between such tumorigenicity and the SV40 TSTA in families of clonal cells with known genealogy.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To elucidate certain biochemical and biological changes in the cell surface and in control of cell division which occur during transformation to malignancy in mouse cells.

Methods Employed: Labelling glycoproteins, glycosoaminoglycans, proteins and glycolipids, also of nucleic acids with radioactive precursors during cell growth in culture. Novel fractionation techniques for isolation of (labelled) subcellular components, including cell surface membranes. Isolation and characterization procedures in thin-layer and column chromatography, gel electrophoresis, electro-focusing and autoradiography of gel slabs. Analytical and preparative ultracentrifuge studies. Enzymology techniques pertinent to intermediary metabolism of carbohydrates. Serum mediated cytotoxicity assays as measured by release of radioactivity from lysed cells. Cell growth dynamics in culture, and also in syngeneic and in nude mice.

Major Findings: I. Changes in cell membrane components in exponentially growing mouse fibroblast cells. Clonal derivatives of mouse embryo fibroblast cell lines were developed, with controlled genealogy and careful matching of sister clones. Sister clones were obtained with differing tumorigenicity (sarcoma) in the syngeneic AL/N or Balb/c mice, including highly tumorigenic cells ($TD_{50}=10^2$ cells/mouse). These cells appeared spontaneously in tissue culture, with no obvious or suspected cause for their malignant transformation (cf. no detectable "C" type viruses). The normal (non-tumorigenic) and the spontaneously transformed tumorigenic clonal cell lines were then infected with SV40 virus, and both SV40 transformed (T antigen positive) and non-transformed reclones were obtained. The various cloned cell lines were then characterized with regard to their growth in the syngeneic and in the nude mice (TD_{50}), and also to their growth properties in tissue culture both in attachment to substratum and in viscous suspension medium independent of anchorage. In one set of studies these biological parameters were then correlated with various biochemical properties of the cell surface membrane.

A. Protein changes. The families of mouse fibroblast cell lines in exponential phase of growth were compared in protein constitution of their cell membranes. In preparations from these cells enriched in cell-surface membrane we observed one protein component (apparent molecular weight about 250,000) consistently to be reduced or absent in an SV40 virus transformed cell line, when compared with the normal cell line. This was in general agreement with the finding of others (Hynes, et al.) on the so-called LETS proteins. However, significantly, no such compositional difference was observed in spontaneously transformed tumorigenic clonal derivative cell lines, or in subclones of such derivative cell lines, with or without SV40 virus infection. However, in metabolic labeling experiments with ^{14}C-labeled mixed amino acids, a consistent decrease also was demonstrated in the biosynthesis of the same protein in the SV40 virus infected subclone, as compared to an uninfected sister subclone, during exponential growth. We showed that this specific difference in biosynthesis is related to the presence and functioning of the SV40 gene, and correlates with the ability of these cells to grow in viscous medium, but, most significantly, *not* with the spontaneously developed high cellular tumorigenicity in the syngeneic animal (AL/N mouse).

Our results are now being confirmed with recent studies of others (some yet unpublished) which also show that spontaneous neoplastic transformation in culture does not lead to the loss of the LETS protein, and transformation induced by chemical carcinogens or by viruses can lead to the loss of the LETS protein in some, but not in all cases of tumor cells (Hynes *et al.*, MIT; Chen *et al.*, CSHL and Harvard). This work in our laboratory by Drs. J. M. Coll, S. J. Pancake and S. W. Luborsky, has now been concluded.

B. *Analysis of various glycoconjugates*. To carefully compare a pair of exponentially growing cell lines in their glycoconjugates, one type of cell was grown with ^{3}H, and the other with ^{14}C glucosamine, under identical conditions. Three culture compartments have been examined; 1) the medium, 2) the material easily released from the cell surface without disrupting or altering the viability of the cells by gentle trypsin treatment, and 3) the trypsinized but still viable cells. The same compartments from the two types of cells were mixed to assure that the differences obtained were due to the different cells, and not to differences in experimental procedures. Each compartment was digested by papain. The macromolecular (non-dialyzable) residues were separated by ion exchange. A computer program and printout was developed (also useful for any other pair of different radioactive labelled components) to give immediate and quantitative comparisons of the two types of cells in the various separated glycoconjugates. The complex mixture of glycopeptides, hyaluronic acid, heparan sulfate and chondroitin sulphate were resolved in the three compartments: the medium, the trypsinate and the trypsinized cell residue.

When comparing biologic phenotypes of 2 arbitrarily selected clones and the parental embryonic mass cell line, the 3 cell lines had very similar *in vitro* growth properties, including their plating efficiency in viscous medium. The tumorigenicity of the cells, determined in syngeneic mice, was found to differ. All 3 cell lines were found to have similar glycoconjugate distributions, although a slight relative increase in labeled hyaluronic acid was found in the more tumorigenic mass cell line than either of the less tumorigenic daughter clones. This difference was most noticeable between those cells whose tumorigenicity differed by the largest amount. The finding implies that increased secretion of hyaluronic acid may benefit tumor growth. This is consistent with some other observations scattered throughout the literature, but not done previously with matched cell pairs from the same family, and by the quantitative and automatically normalizing comparative techniques employed by us. These differences, however, may just be caused by a coincidence in the clones, and further pertinent experiments to tumorigenicity have been carried out by a new design in the biologic approach.

In this approach two independent clonal lines of moderate tumorigenicity (210C, $TD_{50}=10^{5}$, and 216C, $TD_{50}=10^{6.4}$), obtained by picking single cells from the same established mass cell line (201, $TD_{50}=10^{4}$) from the AL/N mouse embryo, were passed through two syngeneic mice, and the two tumor lines were reestablished in culture (220 CT and 219 CT respectively). The tumorigenicity of these resulting two tumor lines increased at least a thousand fold, indicating that from the clones the tumors were induced by selected variant cells with high tumorigenicity ($TD_{50}=\leq 10^2$). None of the above cells were able to grow without anchorage in viscous methocell. These, and other biological observations (also with V. W. McFarland and J. M. Küster) showed that tumorigenicity in the syngeneic mouse and growth without anchorage does not (necessarily) correlate with each other. The elution profiles of labelled glycosoaminoglycans of 220CT and 219CT were essentially identical to their respective parental clonal line of 216C and 210C in all their (cellular) compartments, except a reproducible shift in the elution of the heparan sulfate peak, indicating lower charge density reflecting differences in the metabolism of heparan sulfate (see below) in both of the tumor lines. In light of other findings in this laboratory and elsewhere (see below) this is a significant correlation with the increased tumorigenicity.

In another set of experiments on SV40 transformation, the parent clonal cell line 210C was infected with SV40 and immediately recloned. Two T antigen positive clonal daughter lines (214SCS and 215CSC) which are products of independent transformation events by SV40, and a T antigen negative clonal daughter line (213CSC) were investigated both biologically and biochemically. The tumorigenicity of 214CSC and 215CSC were $\sim 10^{6.5}$; while 213CSC was $\sim 10^{5.5}$. Differences were more significant in saturation density (and possibly in anchorage independent growth), showing significant increase in saturation density in the T antigen positive 214CSC and 215CSC as compared to the T antigen negative sister 213CSC or to the parent 210C. The biochemical correlate was similar to that described above (for the derivative tumor cell lines 219CT and 220CT): a reproducible shift in the elution profile of the heparan sulfate peak towards fractions eluting at lower salt concentration from the anion exchange column, when the $glcNH_2$ labelled compartments from the cell surface trypsinate or that released in the culture medium were compared with the parent 210C. These changes were highly reproducible, were observed during both logarithmic and stationary phase of growth, and neither change was observed in the T-antigen negative sister subclone. The molecular size was unaltered as determined by exclusion chromatography. Ratios of (^{35}S) to (^3H) for heparan sulfate obtained from cells doubly labelled with (^{35}S) sulfate and (^3H) glucosamine were lower in the T-antigen positive subclones. Similar changes for the (^{35}S) to (^3H) ratio of chondroiting sulfate were associated with only small alterations in elution from anion exchange columns. Kinetic experiments suggested a reduced rate of incorporation of (^{35}S) sulfate with no change in turnover rate.

The alterations in heparan sulfate metabolism correlated with the expression of T-antigen and with the cells ability to grow to high densities in monolayer culture, but not with growth in suspension in viscous medium. Tumorigenicity of the subclones was essentially the same as that of the parent clone. These important observations in altered metabolism of heparan sulfate, the significance of such observation to control of cell growth, *in vivo*, and to the possible function of the SV40 T antigen in cell growth in culture are being further pursued. The work is being extended to the SV40 lytic system, and also to new transformant cells obtained by Drs. Seif and Cuzin at the University of Nice, with wild type and temperature sensitive mutants of SV40. (Work of Dr. J. J. Winterbourne)

II. Controls of expression and methods of purification of T, surface, and tumor specific transplantation antigens. The control of the expression of the early (A) gene of a resident SV40 genome in SV40 transformed cells became a center of attention by SV40 virologists and molecular biologists. The A gene has at least two functions: 1) in the one hand it is responsible for the initiation and possibly the maintenance of the transformed cell growth phenotype (as far as certain tissue culture growth properties), 2) it apparently also encodes the nuclear T, and possibly the other SV40 specific antigens, such as the cell surface antigen (TSSA), and the tumor specific transplantation antigen (TSTA).

A) The further progress in detergent solubilization, subcellular localization and partial purification of the SV40 antigens from SV40 transformed mouse cells (with S. W. Luborsky and C. Chang, also in part with R. Weil, University of Geneva) is reported in Project No. Z01 CB 05544-10. In essence the SV40 specific T antigen has been extracted from SV AL/N mouse embryo tissue culture cells by treatment with Triton X-100 detergent. The extracts were found to contain tumor specific transplantation (TSTA), and tumor specific surface (TSSA) antigens, as well. These extracts were purified by ammonium sulfate precipitation, and DEAE- and phsophocellulose column chromatography, and quantitatively assayed for all three antigens. We found that T antigen, TSTA, and much of the TSSA co-purified through all purification steps. This finding is consistent with previous suggestions of the close degree of homology that must exist between the protein species carrying these three antigenic determinants. The antibody-mediated cytolytic assay may detect a new type of antigen on the cell surface, possibly different from T antigen and TSTA: two antigenic fractions were obtained from the phosphocellulose column which had TSSA activity, but one of these did not have T antigen or TSTA activities. The TSTA activity, similar to T antigen, interacted with double stranded DNA. This finding allowed purification of TSTA by DNA columns; also it further supported observations by others that the gene A product has a regulatory role in cell growth in culture (this role, however, may contrast with the role *in vivo*, see below).

B) Further study of the relationships of the T, TSSA and TSTA was carried out in a collaborative work (with C. Chang, S. W. Luborsky, R. Martin, NIAMDD, and D. M. Livingston, Sidney Farber Cancer Center, Boston). The results are reported in Project No. Z01 CB 05080-01 by C. Chang. In short the biochemical and purification studies we found that the SV40 specific T and tumor specific transplantation antigens (TSTA) co-sediment at 6.7s, both precipitate with anti T-serum. A pure protein of 92K molecular weight possesses both antigenic activities. Thus, we demonstrated conclusively that the SV40 A gene product provides the specific transplantation rejection activity in mouse.

C) A completely novel size (56K Dalton) T antigen was found in all (~25) SV40 transformed (cloned) mouse fibroblast cells tested. The status of these findings is also reported in Project No. Z01 CB 05080-01 I by C. Chang. Also in this report can be found other immunochemical approaches on correlating T antigen, TSSA and TSTA in various SV40 transformed mammalian cells (mouse, rat, human), work with SV40 tsA (temperature sensitive) and deletion mutants (in collaboration with Dr. D. Simmons, NIAID, and investigators from the Cold Spring Harbor Laboratory, Drs. Topp and Fey).

D) Work on the unusual processing (transcription and translation) of the SV40 A gene in transformed cells which results in the 56K protein is being done by Dr. G. Khoury of NCI, and Dr. S. Weissman of Yale University Medical School, using cells supplied by us.

E) Spontaneously transformed tumor cells are well known to have low or no detectable TSTA activity. In a few experiments we have attempted,we seem to be able to demonstrate weak TSTA in spontaneously transformed AL/N mouse cell lines.

F) Spontaneously transformed cells, including numerous clones and also clones obtained from tumor lines consistently (11/11) showed a significant (about 100 X) lower tumorigenicity in the nude mouse, than in the syngeneic mouse (AL/N or Balb/c). There was no correlation between tumorigenicity either in the syngeneic or the nude mouse, and the anchorage independence of cell growth. (Work with C. Chang and V. W. McFarland.)

III. Selection of phenotypic and genotypic revertant tumor cells which lose SV40. During our studies of infection of cloned mouse cell lines with SV40, we noticed that, as a rule, if a spontaneously transformed highly tumorigenic mouse cell is infected and further transformed by SV40 virus, the resulting cell has much lower tumorigenicity. The above studies on SV40 specific TSTA explain the reason for these findings. A fortuitous finding of a marker chromosome in the course of the above work in a crucial clone led us to find a reproducible method to select cells which have lost the SV40 antigen expression, by subjecting "doubly transformed" clonal cells to immunologic selection against the SV40 A gene in the immunologically competent AL/N mouse by tumor passage.

A clonal line of highly oncogenic "spontaneously transformed" mouse cells (104) was transformed in tissue culture by SV40 and subsequently recloned. The clone of SV40-transformed cells (106) expressed SV40-specific T and transplantation antigens but was 100 times less tumorigenic than the parent 104 cells. When a relatively large number of 106 cells (10^4-10^5) were injected into syngeneic AL/N mice, tumors were produced. From the tumors, cell lines were established in culture, all of which were consistently negative for T antigen. Tumor lines tested were found not to contain SV40-specific transplantation antigen and had again become highly tumorigenic. The original 106 cells contained about one copy of SV40 DNA per diploid amount of cell DNA, as well as RNA complementary to the early region of the SV40 genome. The T antigen-negative cells from tumor line 124 contained approximately 0.5 copy of SV40 DNA per diploid equivalent and did not synthesize any detectable virus-specific RNA. Reassociation kinetic analysis with restriction enzyme fragments of viral DNA demonstrated that the cells from tumor line 124 (and also the clones of this line) had lost DNA sequences predominantly from the early region of the SV40 genome. The results indicate that a set of stably integrated SV40 DNA sequences can be present in a cell without the expression of viral antigens.

The revertant A gene defective clonal tumor cells can be re-transformed by SV40 and T antigen and TSTA positive cells can be obtained. On transplantation of a T antigen-positive clone, T (and TSTA) negative revertant tumor cell line can be obtained again.

Thus the above method of selection of A gene deleted revertants is a reproducible phenomenon in the AL/N strain mouse. Furthermore, it is not a property of a specific spontaneously transformed cell (such as 104), or a specific transformation event (which resulted in 106 and in further derivative cell lines). An independently obtained clone of AL/N mouse embryo cell line (210C, see above), when transformed by SV40 (215CSC),when passed through the immunologically competent syngeneic AL/N mouse resulted in one T antigen negative tumor cell line (223). However, other T antigen positive tumor cell lines (221, 222, 224) were also obtained in separate mice, when large amounts (10^6 or 10^7) 215 cells were injected. Apparently the immunologic competence of a mouse can be overcome, in which case the mouse is not capable to screen out all of the T and TSTA positive cells and allow growth of only the (rare) T defective revertant cells.

The *in vivo* method described above allowed for the first time selection for revertant cells which lost the early (A) SV40 gene. No such selection occurred in the T cell deficient nude mice (the tumors were T, TSSA and TSTA positive). These experiments open up a new kind of genetic, immunochemical and biochemical study on the mechanism of (spontaneous) loss of a well characterizable gene (the A gene of SV40), apparently by excision and recombination.

Following the above strategy using a Balb 3T12 cell line as a start (obtained from G. Todaro) we have not been able to obtain T or TSTA revertant tumors from Balb/c mice. Up to date 6 tumor lines were obtained when injecting 10^5-10^6 or 10^7 clonal cells transformed by SV40: all 6 tumor lines obtained were T antigen positive. However, the tumorigenicity of the T (and TSTA) positive SV40 transformed cells were always significantly ($\sim$100 X) lower, than that of their parent clonal line. (Work with V. W. McFarland, J. Küster, G. Khoury and C. Chang.)

IV. *Study of the balance between tumorigenicity by spontaneous transformation and the SV40 induced antigenicity*. The above summarized findings on the nature and function of the early SV40 protein is only seemingly paradoxical in the opposing cell growth stimulation function in culture *vs*. their role in recognition and rejection *in vivo*. SV40 virus or SV40 transformed cells are essentially non-tumorigenic in mice, and in the great majority of species tested, including the natural primate host. The SV40 transformed weekly tumorigenic mouse cells likely acquired their tumorigenicity by spontaneous transformation in lengthy tissue culture.

From experiments reported in III, it is clear that immunologic expression of the early A SV40 protein on the SV40 transformed cell surface can cause rejection, and operationally oppose tumorigenicity. In AL/N strain mouse cells which became highly tumorigenic spontaneously, further transformation by SV40 causes reduced tumorigenicity. From such "doubly" transformed cells, early SV40 gene deficient revertant tumor cells can be obtained reproducibly through immunologic selection pressures.

It should be kept in mind that evolutionary selection may favor mechanism of recognition and rejection of tumorigenic viruses and of cells transformed by such viruses in hosts where such viruses occur, especially if endemic. The studies we employed were on, and the majority of tumor (virus) immunology is oriented towards artifactual models, which select those virus host systems for study where transplantation antigens are strong (cf. also current views of G. & E. Klein). There is only weak evidence for host defense against tumors of spontaneous origin.

In extending our studies to numerous clonal mouse cell lines from spontaneous or cell density induced transformations in tissue culture, the importance of somatic mutation(s) in the first step in the (gradual) acquisition of tumorigenicity in a cell became more and more evident. Somatic mutation may thus be the primary event, which may then be enhanced by (a) further mutagenic event(s). Somatic mutation in this sense would include any heritable change in nucleotide sequence, resulting from an alteration, deletion or rearrangement in the primary structure of cellular DNA.

The senior investigator of this project (P. T. Mora) has been given a chance to systematically review in the forthcoming year during a work-study assignment at Oxford the existing evidence supporting the above presumption; to contrast it with evidence that in some cases transformation may be the result in an epigenetic change in the expression of (a) gene, or activity of (a) gene product which is not due to alteration in the primary structure of the DNA. However, he is still not clear in his mind at this time if he can devise feasible experiments in an attempt to prove or disprove the "mutation" theory. Obviously, such exploratory experiments should include determination of 1) frequency of spontaneous appearance of a measurable new phenotype; 2) of increase in 1) mutagenic stimuli; and of 3) reversion frequence. Furthermore, if somatic mutation is the first step in the acquisition of tumorigenicity, then what can one do about it, and if nothing, what can one do about the successive (enhancing) steps, or how to balance or control the progression?

Significance to Biomedical Research and the Program of the Institute: Studies of cell surface biochemical changes which relate to acquisition of tumorigenic potential caused by some rare event such as somatic mutation, and the studies on immunologic recognition and rejection in vivo are of interest, especially when they may lead to understanding of changes in molecular mechanism which appears to be reducible to heritable changes on control processes on the DNA level.

Proposed Course of the Project: There will be an attempt to further clarify the relevance of the various changes in cell membrane biochemistry especially in the heparan sulfate to spontaneous and to viral induced transformation of cells. We also plan to relate these changes to phenotypic changes in cell immunogenicity and in tumorigenicity in various syngeneic, allogeneic and nude mouse systems. Studies on molecular level will also include regulatory events which may be due to association of the A gene product with selective (cf. nuclear membrane associated) portions of the cellular DNA and plans are being made with Dr. S. Shifrin and a group at the University of Connecticut to exploit this lead. We hope that the information and methodology developed in the mouse system will be of use on human tumor cells. Broader plans of P. T. Mora in a possible work-study assignment at Oxford are outlined in Section IV of this project.

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Coll, J. M., Luborsky, S. W. and Mora, P. T.: Metabolically labelled cell membrane proteins in spontaneously and in SV40 virus transformed mouse fibroblasts. Biochem. 16: 3169-3177, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05544-10 I

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Luborsky	Chemist	I NCI
Other:	C. Chang	Visiting Associate	I NCI

COOPERATING UNITS (if any)
R. G. Martin, LMB, NIAMDD

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.2	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study surface changes in transformed cells and the molecular mechanisms responsible for antigenic changes and/or the transformation to malignancy, either spontaneously or as induced by SV40 virus. To develop better methods to isolate and purify cell surface membranes, and antigenic components such as SV40 virus specific surface antigens and tumor specific transplantation antigens; and to study their interrelationships.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: As part of a study of surface changes in transformed cells we wished to develop and improve methods to isolate cell surface membranes, to compare those isolated from normal, spontaneously, and viral transformed cloned and mass cell lines; and to solubilize and characterize the antigens from SV40 transformed cells, and investigate their interrelationships.

Methods Employed: Tissue culture cell cultivation, infection with SV40 virus, cell cloning procedures, polyacrylamide gel electrophoresis, column chromatography, ultracentrifugation, various immunological assays to detect tumor antigens.

Major Findings: The extraction and purification procedure previously developed by us was used to obtain rapidly soluble membrane components from various cultured cells. These included AL/N mouse embryo cell lines, both normal and transformed. Solubilization was generally achieved using 0.5% Triton X-100 detergent in TBS, and was followed by ammonium sulfate fractionation. In some work, 0.5% sodium deoxycholate was also included in the extraction mixture. The extracts from SV40 transformed cells were assayed both *in vitro*, in inhibition of antibody mediated cytolysis (TSSA), and in complement fixation tests (T antigen), and *in vivo*, by immunization of mice against subsequent challenge with syngeneic SV40 transformed tumor cells (TSTA).

The SV40 specific T antigen was extracted from SV AL/N tissue culture cells by a detergent method which also included sonication. The extract was found to contain TSTA and TSSA as well. The 0-60% ammonium sulfate precipitable fraction from these extracts was purified by DEAE- and phosphocellulose column chromatography, and assayed for the three antigens. We found that T antigen, TSTA, and much of the TSSA co-purified through all purification steps. The antibody mediated cytolytic assay appears to detect a new type of antigen on the cell surface, different from T antigen and TSTA:. two antigenic fractions were obtained from the phosphocellulose column which had TSSA activity, but one of these did not have T antigen or TSTA activities. This, with other previous observations, indicates that the TSSA may also contain other antigens which are related to "transformation" but are not SV40 virus coded (e.g., embryonic antigens, etc.).

Further studies of the T antigen and TSTA activities, purified as given above, showed that they co-sediment through a 5-20% sucrose gradient, at 6.7s. Both antigen activities precipitated with anti-T serum; neither, with normal hamster serum; both the T antigen and TSTA activities were recovered in the anti-T serum precipitates. A purified T antigen active protein of 92K molecular weight, homogeneous on SDS polyacrylamide gel electrophoresis, was found to possess TSTA activity as well.

Together with previous demonstrations of co-purification of TSSA and of TSTA by us, these findings are consistent with previous suggestions of the close degree of homology that must exist between the protein species carrying these three antigenic determinants.

We have continued our studies of the binding of these antigens to DNA. This is of particular interest since it implies a possible role in control of DNA function and hence in cell properties. We have available both rapid and quantitative fractionation as well as assay procedures for these early SV40 antigens. These techniques thus can be used to compare and clarify the properties of these antigens and their possible dual role. In addition to acting as antigens, they may possibly act as elements in the establishment, control, or maintenance of the transformed state of a cell. We have observed the binding to DNA cellulose columns of a partially purified preparation containing both T antigen and TSTA activities. This material was extracted originally from SV AL/N cells and partially purified by ammonium sulfate precipitation and DEAE cellulose chromatography. Both activities eluted together from the DNA cellulose column, providing yet another example of the co-purification of these antigens. We hope to study the interaction with DNA of these three antigens isolated from subcellular fractions.

We have fractionated cells to obtain cell membrane, nuclear, microsomal, and cytoplasmic supernatant fractions. Our procedures have been based upon those reported in the literature, and are adapted for our purposes to be simple, rapid and quantitative. When cells are labelled during growth with ^{35}S-methionine and fractionated, virtually 100% of the whole cell radioactivity is recovered in the above four fractions of interest. Radioimmuno-precipitation with antibody and protein A, SDS polyacrylamide gel electrophoresis, and autoradiography were carried out to identify the nature, distribution, synthesis and processing of the early SV40 antigens. Cell fractions prepared by various procedures are being tested by these techniques.

Significance to Biomedical Research and the Program of the Institute: The properties of the cell surface, its chemical composition, structure and antigenic determinants exert important influences upon cell growth characteristics and the ability of certain cells to form tumors or to be rejected by the host. Studies of cell surface biochemical changes which relate to acquisition and expression of tumorigenic potential or to immunologic recognition and rejection *in vivo* are therefore of particular interest. Since we have solubilized three SV40 specific antigens, we can study the correlation of *in vitro* cytotoxicity and complement fixation, and *in vivo* transplantation-rejection assays, as well as the molecular nature of these antigens, their possible interrelationships, and their sites of action in the cell.

Proposed Course of the Project: Extracts will be prepared from whole cells and from the isolated cell fractions, and assayed for SV40 antigens. Isolated antigens will be further characterized by ultracentrifugation as well as gel electrophoresis, to determine more previse values for their molecular weights. Purified antigen preparations will be used to raise antibody for the preparation of affinity columns to more rapidly and quantitatively prepare SV40 antigens for further study. In the light of their co-purification, we will attempt to understand the interrelationships of these early SV40 antigens by investigating events leading to their synthesis and possible processing. The distribution of these antigens in the cell will be investigated in various cell lines. We will attempt to better understand the relevance of the various changes in cell membrane biochemistry and immunochemistry to spontaneous and to viral induced transformation of cells.

Publications

Coll, J. M., Luborsky, S. W. and Mora, P. T.: Metabolically labelled cell membrane proteins in spontaneously and in SV40 virus transformed mouse fibroblasts. Biochem. 16: 3169-3177, 1977.

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Annual Report of the Laboratory of Immunodiagnosis, National Cancer Institute

October 1, 1977 - September 30, 1978

This report summarizes the activities of the Laboratory of Immunodiagnosis for its third full year, since its initiation in December, 1974, and its second year without major changes in senior personnel. The only significant change in senior staff has been the conversion of Dr. William H. West from Research Associate to Senior Investigator. Each of the areas of research in this laboratory has been highly productive during the past year: There have been extensive efforts toward characterization of natural killer (NK) cells in mice, rats and humans. Interferon has been shown to play a central role in activating NK cells and K cells mediating antibody-dependent cell-mediated cytotoxicity (ADCC). A considerable focus has been on the role of macrophages, as well as NK cells in mediation of anti-tumor resistance. The opposing, suppressor role of macrophages has also been evaluated. Protocols have been initiated to examine the relative in vivo roles of NK cells and macrophages, and their mutual interactions, in both animal systems and in patients with cancer. A human lung cancer associated antigen has been purified and isolated, and progress has been made in setting up a radioimmunoassay which may be useful in the diagnosis and monitoring of lung cancer. There has continued to be substantial progress in the development of assays of cell-mediated and humoral anti-tumor immunity. These have included advanced technology for migration inhibition assays, a clinical assay for the cytostatic effects of monocytes, and a simplified and sensitive assay for detection of cell surface antibodies. Much additional information has been analyzed on the relationship of clinical course of disease in cancer patients to the results of various assays of immunologic competence in anti-tumor immunity. A collaborative clinical randomized immunotherapy protocol in lung cancer patients is now showing significant prolongation of disease-free interval among patients receiving intracutaneous BCG.

In addition to the research activities of the Laboratory, which are summarized in more depth below, Drs. Herberman and McIntire have continued to be extensively involved in the National Cancer Program activities related to immunodiagnosis of cancer. Both served on the Committee on Immunodiagnosis of Cancer and other contract review committees. Dr. Herberman has been the main focus within the National Cancer Institute for problems related to immunodiagnosis, and he has been particularly involved with the evaluation of many potentially useful serum immunodiagnostic tests, by the use of coded serum panels provided to investigators from the NCI-Mayo Clinic Serum Bank. Drs. Herberman and McIntire have organized an international workshop on Immunodiagnosis of Human Cancer, to be held in Bethesda in September 1978, to discuss in detail the status of immunologic assays which may be used for the diagnosis and/or management of cancer patients.

Because of the limited space and technical staff which have been available to the laboratory, its activities are substantially augmented by support contracts with Litton-Bionetics, Inc., Kensington, Maryland. An additional support contract, for large scale separations of human tumor associated antigens, with George Washington University Medical Center, has recently been phased out, as the laboratory has gained the capability to handle this project intramurally. These support contracts permit more extensive studies of *in vivo* tumor immunity in experimental mice and rats, and the performance of *in vitro* assays of immunity of cancer patients. These support contract projects are performed in very close association with the intramural activities of the laboratory.

The research activities of the Laboratory of Immunodiagnosis can be divided into three main areas: Cell-mediated immunity studies in animal tumor systems, cell-mediated immunity studies in man, and serologic and immunochemical studies of tumor-associated antigens and of lymphokines. The main emphasis in each of these areas is on the application of the research information to the diagnosis of cancer and monitoring of disease in tumor-bearing individuals.

Some highlights of achievements in the past year:

I. Cell-Mediated Immunity Studies in Animal Tumor Systems.

Extensive studies have been continued on the cell-mediated immune response of C57BL/6 mice to inoculation with murine sarcoma virus (MSV). During the past year, there has been considerable attention focussed on activities of macrophages in this system, both in regard to their direct anti-tumor effects and to their ability to help or suppress immune responses.

Macrophages recovered from the spleen, peritoneum or from the tumors of mice injected with the regressor stock of MSV were nonspecifically cytolytic as measured by an 18 hour ^{51}Cr release cytotoxicity assay. Studies conducted during the past year have concentrated on the macrophage-mediated cytotoxicity of cells isolated from tumors induced either in conventional or nude mice by the injection of regressor or progressor MSV virus. Cytolytic macrophages recovered from the tumors of mice injected 14 days earlier with either the regressor or progressor stock of MSV had comparable levels of cytotoxicity. However, macrophages from progressively growing tumors, at 50-60 days after inoculation of the virus, had lower levels of activity. Nude mice on the other hand did not have cytolytic macrophages in their tumors. Other differences were noted in the macrophages derived from the different animals when 1 g velocity sedimentation studies were performed on cells isolated from the tumors. In the tumors from regressor mice, there were two peaks of reactivity corresponding to the main peaks of macrophages identified by latex particle ingestion. However, in the progressors, there was functional activity only in the peak of smaller macrophages. Nude mice, even after separation by velocity sedimentation, exhibited no macrophage-mediated cytolysis.

We found that LPS could enhance macrophage-mediated cytotoxicity when added directly to the assay even at very low concentrations (1 ng/ml). However it appeared that addition of LPS to the assay was only augmenting the reactivity of the cells that were previously exhibiting cytotoxic reactivity and not activating cells that had no reactivity. Several other agents were tested for their ability to enhance macrophage-mediated cytotolysis. We found that poly I:C and muramyl dipeptide, a synthetic preparation of a constituent of BCG, could enhance reactivity as well as LPS, while dextran sulfate and zymosan had wead stimulatory effects. Addition to the assay of a crude preparation of interferon derived from fibroblasts caused similar dose-responsive stimulation of cytolytic activity.

Spleen cells from MSV immune mice could be stimulated in vitro to undergo a secondary cytotoxic response. Low levels of proliferation were associated with this response and by inhibiting proliferation with a hot thymidine pulse the generation of reactivity was almost completely blocked. Also, if macrophages were extensively depleted before the cells were stimulated, no cytotoxicity was detected at the end of the culture period. The response could be restored by adding back syngeneic macrophages to the depleted spleen cells before they were stimulated but not by the addition of allogeneic macrophages or 2-mercaptoethanol. Similar macrophage requirements for the generation of a primary allogeneic cytotoxic response could also be shown. However, in this system, allogeneic macrophages could serve as the source of antigenic stimulus in the absence of syngeneic macrophages. For the syngeneic response to tumor antigens, viable macrophages were required. However, macrophages that had been treated with mitomycin C to block replication were able to function as helper cells in this assay. Mapping studies indicated that similarity at the major histocompatibility complex was essential for the macrophages to act as helper cells and as previously shown for the macrophages needed to help in the stimulation of MIF production in response to soluble tumor antigen, compatibility at the IA subregion appeared to be required. Spleen cells from mice that had progressively growing tumors responded very poorly when stimulated in vitro. However, if the macrophages were depleted and normal, syngeneic macrophages were added back, significant levels of reactivity were generated. If spleen cells from mice with a progressively growing tumor were added to a secondary response culture of spleen cells from regressor mice, the generation of the cytotoxic response could be inhibited, indicating the role of suppressor macrophages in this system.

Studies have been continued on the relative role of T cells in macrophage-mediated inhibition of tumor cell growth in vitro and in suppression of mitogen induced lymphoproliferation. Spleen cells from nude mice injected with C. parvum or MSV were able to suppress the LPS response in nude or conventional mice and they were able to suppress the in vitro proliferation of RBL-5 tumor cells.

Studies have recently been undertaken to explore the possible mechanisms by which macrophages may mediate their suppressor acitivity. Indomethacin, an inhibitor of prostaglandin synthesis, has been used in experiments to determine whether macrophages are mediating their suppression through the secretion of prostaglandins which may be inhibitory. In studies using 1 ug/ml indomethacin in assays of suppression of LP responses to mitogens in mice, the indomethacin was only effective when low levels of suppression were present.

We have continued to perform extensive studies on natural cell-mediated cytotoxicity against tumor cells. During the past year, our studies have focussed on the following areas: a) Factors affecting the levels of natural killer (NK) activity in rats and mice; b) the immunopharmacology of NK activity in rats and mice; c) the mechanism of NK activity, particularly the relationship to antibody dependent cell-mediated cytotoxicity; d) relationship of NK activity to in vivo resistance to tumor growth. It has been possible to augment the levels of NK reactivity in young mice and rats, or to cause a reappearance of NK reactivity in older mice or rats, by inoculation of cells bearing the relevant antigens or non-specifically by inoculation of various rodent viruses, BCG or Corynebacterium parvum. All of these treatments produced a peak of cytotoxicity at 3 days which was detectable in all of the lymphoid organs, except the thymus. The effector cells mediating the boosted cytotoxicity had the same characteristics as those of NK cells in unstimulated animals. Poly I:C, pyran, endotoxin, and several other interferon inducers were administered to mice and all of these produced augmentation of NK reactivity, with peak activity at about the time of known peak levels of interferon. Administration of crude and purified preparations of interferon prepared in fibroblasts caused very rapid boosting, with a peak at about 3 hours. In addition, incubation of normal spleen cells with poly I:C overnight, or with interferon preparations for as short a period as 45 minutes, resulted in substantial increases in NK activity. When carrageenan or silica were given prior to administration of poly I:C, boosting of NK activity was substantially inhibited. In contrast, when these agents were given after poly I:C and just prior to assay, they had no detectable effects. These results suggested that macrophages might be important cells for production of interferon in response to poly I:C. This was confirmed by direct measurements of interferon levels in the serum of mice. In addition to the studies related to macrophages, the effects of NK activity of a variety of immunopharmacologic treatments were examined. In both rats and mice, spontaneous levels of NK activity were shown to be inhibited by treatment with hydrocortisone, cyclophosphamide, or lethal X-irradiation. In contrast, these treatments had no detectable effect when administered prior to boosting with poly I:C. These findings led to the hypothesis that NK cells develop from precursors which are substantially more resistant to X-irradiation and cytotoxic drugs. The differentiation from pre-NK cells to functionally active NK cells appeared to be independent of proliferation, since treatments which inhibit DNA synthesis, including methotrexate, X-irradiation, and cytosine arabinoside had no effect on the ability of animals to be boosted.

Studies of immunity of rats to the W/Fu Gross virus induced lymphoma, (C58NT)D, have continued. The principal focus during this past year has been on the production of migration inhibitory factor (MIF) in response to these tumor cells. A major finding, which may be analogous to the natural cell-mediated cytotoxocity in rats, has been that normal W/Fu rats, particularly those greater than 6 weeks of age, have the ability to produce MIF upon incubation with the established tissue culture cell line of (C58NT)D. As a possible further analogy to rat NK reactivity, BN rats, which have low NK reactivity, have also been found to have little or no ability to produce MIF in response to these tumor cell lines. A further characteristic of this response has been the requirement for histocompatible macrophages, both in response to intact tumor cells as well as to 3M KCl extracts of tumor cells.

Direct agarose microdroplet LMI assays were used to monitor systemic immunity against line 10 extract (TAA) and PPD in a strain 2 guinea pig immunotherapy model. Animals sensitized to only BCG became reactive to TAA while animals sensitized to line 10 reacted to PPD, suggesting common antigens between line 10 and BCG. When treatment groups were compared, LMI appeared predictive of prognosis. Groups with large tumor burdens and having a poor prognosis did not respond as well in LMI against TAA as did groups having favorable prognosis.

Direct macrophage migration inhibition micro-assays were used to monitor TAA activity of preparations of SV40-induced mKSA cells, the mineral oil induced ADJ-PC5 plasmacytoma, and the methylcholanthrene-induced Meth A tumor. The antigen preparations included cell-free crude membranes, papain-solulized and NP40 detergent-solubilized membrane extracts. The assay was extremely sensitive and could detect migration inhibition reactivity with all three types of antigenic preparations, with picogram concentrations.

We have investigated the development of cellular immunity to MuMTV in normal virgin C3H+ and genetically similar C3H- female mice by the indirect agarose droplet migration inhibition assay. Spleen cells from C3H+ mice were able to respond to MuMTV antigens and produced a factor (presumably macrophage migration inhibition factor, MIF) that inhibited migration of normal mouse peritoneal exudate cells (PEC). This reactivity appeared at 14 weeks of age in C3H+ mice but was no longer detectable at 36 weeks. In contrast, reactivity was never displayed by spleen cells of C3H- mice. Injection of 10 μg MuMTV in C3H+ or C3H- mice younger than 14 weeks of age induced similar reactivity to MuMTV antigens in both mouse strains. This pattern of reactivity suggested that normal C3H+ mice are not tolerant to antigens of MuMTV and that overt infection is required for induction of an immune response. A response to MuMTV antigens could be detected in C3H- mice younger than 14 weeks of age which were bearing transplantable mammary tumor, but C3H+ tumor-bearing mice were generally unable to respond to these antigens. Normal BALB/c mice developed migration inhibition reactivity to MuMTV if they were housed for several weeks in the same room as MuMTV-bearing mice, suggesting that horizontal transmission of the virus is involved in the immunobiology of MuMTV. Although C3H+ mice bearing transplantable syngeneic mammary tumors were generally unable to produce MIF in response to MuMTV, a strong immune response in those mice was elicited by a 3M KCl extract of MAT-2 tumor. Furthermore, a 3M KCl extract of C3H+ embryos, with no detectable MuMTV antigens, was found to stimulate migration inhibition reactivity in C3H+ and C3H- mice bearing MAT2 tumor, suggesting that tumor growth induces reactivity against a non-virus-related TAA which is common to embryo cells. Induction of MIF production by MAT-2 TAA or MuMTV appeared dependent on T cells, since reactivity was eliminated by pretreatment of immune spleen cells with anti-θ + complement.

The migration of PEC from endotoxin-unresponsive C3H/HeJ mice in response to MIF was compared to that of equal numbers of PEC from closely related, endotoxin responsive, C3H/HeN mice. Macrophage migration with PEC from C3H/HeN mice was strongly inhibited (less than 50% of control) through a 1/32 dilution of MIF-supernatants. In contrast, no migration inhibition was detected with PEC from C3H/HeJ mice even with undiluted MIF-supernatants. Macrophages from C3H/HeJ became responsive to MIF after the hosts were pretreated with BCG. Furthermore, PEC from C3H/HeN mice admixed with 5-10% PEC from C3H/HeJ mice became completely unresponsive to MIF-supernatants. These results indicate that C3H/HeJ mice don't have an intrinsic defect in their macrophage responses to MIF, but rather that some reversible form of suppression exists.

II. Studies of Cell-Mediated Immunity in Man.

We have continued to study in detail the characteristics of human NK cells, with emphasis on their lineage and on their relationship to K cells mediating ADCC. The NK cell is an FcR+ lymphocyte of the T cell lineage, inseparable from and perhaps identical with the killer (K) cell that mediates ADCC in the well-defined Chang liver cell system. This year we were able to confirm the T cell lineage of the NK and K cells by using a specific anti-T cell serum. Treatment of effector cell populations with anti-T cell serum and complement resulted in abrogation of NK and K. The anti-T cell serum reacted not only with sE-RFC but also with a large proportion of the non-sE-RFC found in the interface after sheep erythrocyte rosette cell separations, that was also responsible for the NK activity of the interface.

The evidence for the identity of NK and K Cells has come mainly from cold target cell inhibitions of the CRA. It was possible to inhibit ADCC reactions with NK target cells, like E-14 and K-562, but not with target cells resistant to NK, e.g., Chang liver cells.

Whereas most investigators agree on the similarities between the NK and K cells, there is much controversy as to whether NK activity involves the FcR and IgG antibodies. During the period under review we have accumulated several pieces of evidence showing that IgG antibodies attached to FcR play no role in NK: 1) Although one laboratory has claimed that NK was inhibited by monovalent fragments (Fab fragments) of goat and rabbit anti-human IgG, we have not been able to reproduce these results with a monovalent Fab fragment of goat anti-$F(ab')_2$ reagent. 2) Although a different laboratory has claimed that NK was due to K cells armed with natural IgG antibodies, as shown by specificity testing and rearming of trypsinized effector cells, we were unable to reproduce these findings, even after an extensive series of attempts under various conditions. 3) We have established that several immune complexes that completely block ADCC do not block NK. 4) After elution of cytophilic IgG from FcR by incuba-tion at 37° or by washing, NK has remained stable. 5) In studies on the generation of NK *in vitro*, B cells and/or antibody production were not needed to generate NK activity.

Further interesting data were obtained with our model of *in vitro* generation of NK and K cells. If MPBL are first depleted of FcR+ cells, to deplete their NK and ADCC, these activities reappear in cultures with fetal bovine serum, or with K-562, within 5-7 days. During the period under review, we particularly analyzed the cellular requirements for the regeneration of these activities. Sheep erythrocyte fractionation studies revealed that neither the E-RFC pellet nor the non-E-RFC interface were able to regenerate much activity. However, when both E-RFC and non-E-RFC were mixed together good regeneration occured. It was possible to generate activity after X-irradiation of the E-RFC fraction but not when the non-E-RFC were irradiated. Within this latter fraction, the null cells, rather then the B cells, were found to be required for generation of NK and K cells. The specificity of the NK activity regenerated after 7 day culture of MPBL was also examined and compared to that of the same fresh, normal MPBL tested on day 0, by cold target cell inhibition in the CRA. In most instances, the specificity after regeneration on day 7 was very similar to that observed with the MPBL on day 0. This shows that the activity on day 7 of culture is that of NK cells, and strongly supports our contention that our culture system represents an *in vitro* model for the generation of this activity.

Similar to the demonstration of the activation of mouse NK cells by interferon, we found that purified interferon preparations from lymphoblastoid cell lines, fibroblasts, or lymphocytes were able to boost preexisting NK and K cell activities. However, interferon did not appear to facilitate the development of FcR- precursor cells into FcR+ effector cells. Anti-interferon was able to completely block the activating effects of interferon. Also, we have recently begun to monitor patients receiving poly I:C-polylysine complex, and already have evidence that the levels of NK and ADCC activities are boosted at 2 days.

Our studies on the generation and continued growth of pure T cells have continued. Human cultured T cells (CTC) will grow for at least 3-4 weeks when in the presence of 10-20% of a 4-fold concentrated, commercially available conditioned medium from pooled, allogeneic, PHA-stimulated MPBL. CTC possess many of the typical characteristics of isolated T lymphocytes. CTC will allow crucial studies of the functional and biochemical properties of human T cells, that are uncontaminated with other MPBL. Obtaining such large numbers of cells is extremely dependent on very effective conditioned media, which at present are not routinely available. We have studied the conditions required for production of the mitogenic factor (MF) in conditioned media, by MPBL from single donors, using PHA or Con A, and various concentrations of pooled human serum. Preliminary experiments indicating better and more sustained production of MF in the presence of 10^{-6} M indomethacin or after removal of monocytes support the possibility that prostaglandins from monocytes may be the regulatory mechanism. Also, PHA-transformed blasts in these cultures readily remove the MF activity from the conditioned media. This phenomenon was avoided by 2000 R X-irradiation of the responding MPBL: production of MF occured normally and the cells did not remove activity out of the conditioned media. Our data suggest a very interesting two-cell model of MPBL activation by mitogenic lectins: First, lectins activate some T cells to produce MF. This phenomenon does not involve DNA synthesis and is a radioresistant. Second, a very minor population of MF-receptor positive T cells in MPBL, or perhaps only some T cells that have acquired a MF-receptor after contact with the lectin absorb the MF and enter mitogenesis.

In studies of the functional activities of CTC, they were shown to undergo a proliferative response to allogeneic leukocytes. CTC consistently showed an early (day 2-4) ^{3}HTdR incorporation response to allogeneic cells, and did not respond to autologous cells. An HLA typed family showed that CTC reactivity was determined by antigens coded for by the major histocompatibility complex.

CTC proved to be quite cytotoxic against a wide variety of tumor target cells and against allogeneic PHA-blasts in a 4 hr CRA. This cytotoxicity was much enhanced by the addition of lectins, PHA or Con A. In the absence of lectins, the CTC killed all allogeneic PHA-blasts that were also killed by specific MLC-CML reactions, using MPBL responders from the CTC donor, and they never killed autologous PHA-blasts. Thus, by all criteria found in the literature, the CTC appear to be polyclonal, activated T cells.

Extensive studies have been performed, which demonstrate that CTC act as suppressor cells in at least three different systems: antibody production _in vitro_, proliferative responses to Con A, and proliferative responses in MLC. Although these activities need to be further characterized, and the responsible T cell subsets isolated, this again points to the CTC being an interesting source of functional subsets of T cells.

We have continued our studies on cellular immune competence in cancer patients. Patients with lung and breast cancer, who showed evidence of depressed proliferative responses to PHA, Con A and MLC were systematically recalled, and their MPBL were analyzed for evidence of suppressor cells. In analogy with work done in mice and rats in our laboratory, MPBL were passed over G-10 Sephadex columns to remove monocytes. Under these circumstances the lymphocyte proliferative responses often returned to normal values in lung cancer patients, suggesting an adherent suppressor cell. Indeed, unseparated MPBL or cells adherent to the G-10 columns were able to suppress the responses of normal individuals to PHA, Con A, or in MLC. In cancer of the breast, some patients had suppressor cells that were not removable by the G-10 columns.

Our previous studies with the 29° rosette assay in lung cancer patients suggested that it might provide useful prognostic information. We have therefore performed a detailed analysis on the Stage I lung cancer patients on the randomized immunotherapy protocol. Rosette levels were determined on most patients on a monthly basis and the data were analyzed to determine whether the proportion of depressed rosette values during the first six months after surgery could predict subsequent clinical course. Among patients with 1/3 or more of their tests depressed during this period, a higher proportion relapsed than did those patients who had a lower percentage of depressed tests and this difference approached statistical significance. These results have suggested that repeated rosette testing on lung cancer patients during the first few months after surgery might help to more accurately stage the patients. When the results of the first test performed on Stage I lung cancer patients in the rosette assays were combined with the results of lymphoproliferative responses to PHA and MLC, the results from this single time point provided essentially equivalent discrimination to that obtained by sequential testing by either the 29° rosette assay or the lymphoproliferation assay.

Lung and breast cancer patients were tested for the proliferative responses of their MPBL to autologous tumor material. We could confirm that 60% of our test with lung cancer and 40% with breast cancer patients resulted in clearcut proliferation and the antigens recognized appeared to be tumor associated. Low concentrations of hypotonic membrane preparations worked particularly well in this system. It could be demonstrated that LIF is also produced in these cultures.

Considerable progress has been made in the past year in improving the measurement of leukocyte inhibitory factor (LIF), produced in response to PPD or tumor antigens. In a direct agarose microdroplet migration inhibition assay, inhibition of migration of leukocytes from some PPD skin reactive donors was obtained with as little as 5 x 10^{-3} -- 5 x 10^{-7} μg/ml. Comparative experiments of the agarose micromethod and the capillary tube technique indicated that the agarose assay was more sensitive in that it could detect reactivity with 2-4 logs lower antigen concentrations. Indirect assays using supernatants of cultures of PPD triggered mononuclear cells and indicator granulocytes in agarose droplets demonstrated that the reactions were due to production by immune lymphocytes of LIF.

In addition to developing the direct migration inhibition assay at a more micro-level, we have been interested in generating supernatants with high titers of LIF to be used in indirect migration tests. We have found that supernatants containing high titered LIF (10^{-6} - 10^{-7} dilutions still reactive) can successfully be generated with mononuclear cells from PPD skin-reactive human donors in response to PPD, if the super-natants are made in 50 ml polystyrene conical tubes. During the performance of this study we also observed that some normal donors' polymorphonuclear cells were not sensitive to LIF. The reason is not yet understood, but the finding points out the necessity of testing supernatants with only reactive donor polymorphonuclear cells to eliminate negative tests. Physicochemical studies using Sephadex G-200 columns of these supernatants indicate that the reactive molecule(s) was in the molecular weight range (69,000) of LIF as earlier reported by Rocklin. Further supporting evidence that we are truly dealing with the lymphokine, LIF, in these supernatants was obtained by sugar inhibition studies. Both α L-fucose and N-acetyglucosamine, sugars previously reported to block activity on human polymorphonuclear cells, inhibited the activity of our supernatants, whereas glucose and several other sugars had no inhibitory activity on the LIF containing supernatants.

Indirect migration inhibition tests with lung cancer patients receiving no radiotherapy or other treatment were tested with 3M KCl extracts of fresh pleural effusion cells from a patient with adenocarcinoma of the lung (7661) and of tissue culture cells (CaLu) derived from a Squamous cell carcinoma of the lung. 60-70% of the untreated patients responded, whereas most patients receiving radiotherapy were not capable of producing LIF to these antigens. Of considerable interest, was the observation that many normal donors in close contact with lung cancer patients or with tumor or blood specimens produced LIF in response to these antigens, whereas non-contact donors were unreactive. Experiments during the last 6 months were performed with microfuge tubes, using 5×10^5 mononuclear cells to generate LIF. Reactive supernatants could usually be diluted to an endpoint of 1:100 - 1:1000, which may allow for future semiquantitation of patient responsiveness to these antigens.

Breast cancer patients, normal donors, and benign breast disease patients were tested in indirect migration inhibition assays with a 3M KCl extract of MCF-7, and with MuMTV or its major glycoprotein, gp52.

Recently, in analogy to our studies in rodents, we have developed an assay for measurement of the cytotostatic effects of human monocytes in the peripheral blood. We have found that peripheral blood mononuclear cells from the majority of cancer patients tested, particularly those with advanced disease or receiving BCG, had increased cytotstatic activity against a cultured cell line. This activity was shown to be dependent on adherent and phagocytic cells and therefore appears to measure monocyte reactivity. Recent studies of patients being given poly I:C have indicated marked increases in this activity within 24 hours after inoculation. It seems likely that this new assay will be quite useful in measuring activation of human peripheral blood monocytes, either related to disease status or to immunotherapy.

We have been assessing the possible benefits of post-operative adjuvant immunotherapy with BCG and allogeneic tumor-cells, or BCG alone in a randomized controlled trial for stage I lung cancer patients. Preliminary results indicate a significant benefit of the BCG immunotherapy in terms of disease-free interval. The addition of allogeneic X-irradiated tumor cells had no influence on the results. The favorable results with patients with localized disease using BCG are in contrast with our earlier observations of no effect on patients with more advanced disease.

III. Serological and Immunochemical Studies of Tumor-Associated Antigens and of Lymphokines.

We have developed a more sensitive radioimmunoassay (RIA) for AFP which has reduced the background so that normal AFP serum levels can clearly be measured. This same RIA for AFP has been used to determine quantitative levels of serum AFP in patients with hepatocellular carcinoma. Collaborations with several institutions in this country and abroad are aimed at the monitoring of therapy in these patients. Patients with elevated levels of serum AFP who demonstrated a response to therapy had a concomitant fall in AFP and this was generally a more sensitive and more rapid indication of response than were the usual clinical parameters. Furthermore, the AFP level usually provided the first evidence of resistance to the chemotherapy or to recurrent tumor growth. An advantage of the more sensitive RIA is the ability to more accurately follow changes in serum AFP which are close to the normal level. This has allowed the monitoring of patients with non-malignant liver disease, to determine the clinical correlations with serum AFP in acute infectious hepatitis and in chronic cirrhosis.

Continuing carcinogenesis studies in 1-nitrosodiethylamine (DENA) treated monkey have used serum AFP to monitor induction of hepatocellular carcinomas. Monkey (Mk AFP) was purified from both fetal and hepatoma sources and demonstrated no detectable immunological or physicochemical differences. Similarly isolated human AFP had similar physicochemical characteristics to the Mk AFP, but when compared in the RIA for human AFP showed marked antigenic dissimilarities.

A human lung tumor associated antigen (LTAA-2) was purified from the saline extract of an adenocarcinoma of the lung, using standard biochemical procedures after the addition of a trace quantity of radiolabelled LTAA from a different tumor to facilitate recovery of the antigen. Following isolation of adequate amounts of LTAA-2, a double antibody RIA was set up. In preliminary results comparing normal sera with sera from patients with active lung cancer, there appeared to be a considerable difference in the quantitative levels of these two groups. Unfortunately, there is material in normal serum that competes for binding with the LTAA-2 and the material is probably present in all human sera, cancerous as well as normal. Further refinement of the RIA will require identification of the serum competitor and exclusion or removal of this effect.

The technique of immunoperoxidase cellular staining has been shown to correlate well with elevations of serum markers in germ cell testicular tumors. A preliminary survey of 36 lung cancers with 5 different antisera have shown that all were stained with anti-CEA and 32 with anti-LTAA-2. Only 6 of 18 squamous cell carcinomas and 2 of 4 large cell carcinomas were stained with anti-ACTH; none of the 6 adenocarcinomas or 8 small cell carcinomas were positive with this reagent. Anti-HCG stained only one squamous cell carcinoma and the anti-AFP did not stain any of the lung cancers.

To identify and isolate antibodies to human cancer cell surface antigens, a radiobinding assay was developed. Target cells were fixed in 3% glutaraldehyde and antibody binding was quantitated with radioiodinated staphylococcal protein A. Xonogeneic antisera have been identified which show significantly more binding to lung cancer cells than to normal cells.

Efforts have also been directed toward the isolation of surface antigens from a mouse virusinduced leukemia, MBL-2. NP-40 solubilized materials from surface radio-iodinated MBL-2 were reacted with a variety of antisera to murine leukemia viral proteins (gp 69/71, p30, p15, p12 and p10) produced in rabbit and goats with sera from C57BL/6 mice which rejected a murine sarcoma virus (MSV)-induced tumor, and with rabbit anti-MBL-2 serum. The resulting radio-precipitates were analyzed and compared by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Anti-gp69/71 was the only antiviral protein sera which was active, detecting a "doublet" peak of 70,000 and probable degration fragments of 42,000 and 35,000 daltons. Anti-MSV showed a pattern very similar to that seen with anti-gp69/71, in-dicating probable reactivity with viral envelope antigen, gp69/71. Anti-MBL-2 reacted with a cell surface antigen of 95,000 daltons which did not appear to be related to virion antigens.

With the development of more sensitive and quantitative assays to measure MIF and LIF, we have initiated immunochemical studies of these lymphokines. Fucose affinity columns have been used for the preliminary enrichment of MIF activity. Fucose affinity columns have also been used for enrichment of LIF activity from the supernatant fluid from cultured lymphocytes induced to produce LIF. By sieving chromatography on Sephadex G-200 the LIF activity was slightly smaller than serum albumin, which agrees with other reports. Studies are in progress to define the carbohydrate binding characteristics of LIF so that more specific and/or efficient methods of isolation might be utilized.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05557-10 LID

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cellular Immune Responses in Experimental Tumor Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

Litton-Bionetics, Inc.; University of Perugia, Italy.

LAB/BRANCH
Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural cell-mediated cytotoxicity of mouse and rat lymphocytes against tumors were studied in a short-term ^{51}Cr release assay. NK activity was strongly boosted by inoculation of viruses, immune adjuvants, interferon inducers, and by interferon itself. Interferon appears to play a central role in activating NK cells, and the in vitro effects of interferon or poly I:C could be blocked by the addition of anti-interferon. The response to poly I:C appeared to be mediated by the macrophage-dependent production of interferon, and this mechanism appears to account for the inhibitory effects of carrageenan and silica on NK activity. From studies with a variety of immunopharmocologic agents, it appeared that NK cells are derived from drug and radioresistant pre-NK cells. The differentiation from pre-NK cells to activated NK cells appeared to be independent of proliferation. The mechanism of NK activity appeared to be independent of antibody dependent cell-mediated cytotoxicity, although the levels of NK and K cell activities have correlated closely. In addition to natural cell-mediated cytotoxicity, normal mice from some strains of rats have been shown to produce migration inhibitory factor in response to some virus induced tumor cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The objectives of this project are: 1) To study natural cell-mediated immunity to rodents and to determine its characteristics and its role in resistance against tumor growth; 2) to induce and measure cell-mediated immune reactions against tumor antigens, and to determine the role of various phases of the immune response in resistance against tumor growth; and 3) to analyze the role of lymphoid cell subpopulations in the immune response, and in the suppression of, the immune response to tumor antigens.

Methods Employed: Lymphoid cells are obtained from tumor-bearing, immune and normal subjects. A variety of normal and tumor cell lines are maintained in tissue culture. Inbred strains of mice and rats, and transplantable tumors induced by viral, chemical or unknown agents are used in model systems. Cellular immune reactions to tumor antigens are measured by migration inhibition and lymphocyte-mediated cytotoxicity assays. Subpopulations of lymphoid cells are prepared by adherence to columns and plates, and by treatments with antisera, silica, carrageenan, or other agents. Effects of a variety of immunopharmacologic agents are tested, including immune adjuvants, interferon inducers, interferon, cytotoxic drugs, and X-irradiation.

Major Findings:

I. Natural Cell-Mediated Immunity Against Tumor Cells

We have continued to perform extensive studies on natural cell-mediated cytotoxicity against tumor cells. During the past year, our studies have focussed on the following areas: a) Factors affecting the levels of natural killer (NK) activity in rats and mice; b) the immunopharmacology of NK activity in rats and mice; c) the mechanism of NK activity, particularly the relationship to antibody dependent cell-mediated cytotoxicity; d) relationship of NK activity to *in vivo* resistance to tumor growth.

Spontaneous expression of NK reactivity in mice and rats is quite transient, being detectable at only 5-10 weeks of age. However it has been possible to augment the levels of NK reactivity in young mice and rats, or to cause a reappearance of NK reactivity in older mice or rats, by inoculation of cells bearing the relevant antigens or non-specifically by inoculation of various rodent viruses, BCG or *Corynebacterium parvum*. All of these treatments produced a peak in cytotoxicity at 3 days which was detectable in all of the lymphoid organs, except the thymus. The effector cells mediating the boosted cytotoxicity had the same characteristics as those of NK cells in unstimulated animals. Some insight into the mechanism of boosting was first provided by an observation that administration of poly I:C, a potent interferon inducer, to rats caused more rapid boosting in cytotoxicity, with a peak at 12-18 hours. This raised the possibility that all of the boosting that we had seen was due to production of interferon. Extensive studies were therefore performed in mice to determine the possible role of interferon in regulating the levels of NK activity. Poly I:C, pyran, endotoxin, and several other interferon inducers were administered to mice and all of these produced augmentation of NK reactivity, with peak activity at about the time of known peak levels of interferon. Administration of crude and purified preparations of interferon prepared in fibroblasts caused very rapid boosting, with a peak at about 3 hours. To more definitively examine the role of interferon, a completely in vitro system was developed. It was found that incubation of normal spleen cells with poly I:C overnight, or with interferon preparations for as short a period as 45 minutes, resulted in substantial increases in NK activity. To confirm that the effects seen were due to interferon, we examined the effects of addition of specific anti-interferon. This caused complete abrogation of the augmentation.

Once the role of interferon in augmenting NK reactivity was established, we initiated studies to determine the cells which might be involved in the augmentation. Our efforts focussed primarily on macrophages, since earlier experiments had indicated that administration of macrophage-toxic agents, carrageenan or silica could cause substantial decreases in NK activity. When these agents were given prior to administration of poly I:C, boosting of NK activity was substantially inhibited. In contrast, when these agents were given after poly I:C and just prior to assay, they had no detectable effects. These results suggested that macrophages might be important cells for production of interferon in response to poly I:C. This was confirmed by direct measurements of interferon levels in the serum of mice. To further examine this question, the effects of depletion of macrophages, or of the addition of silica or carrageenan to spleen cells, were examined. When spleen cells were first treated by these procedures and then poly I:C was added, little or no boosting of reactivity was seen. In contrast, these treatments had no effect on the spontaneous NK activity or on NK activity after boosting had been performed. Boosting of NK activity in vitro by interferon was not affected by removal or inactivation of macrophages. Taken together, these results indicate that macrophages are required for the production of interferon in response to poly I:C. It now seems likely that macrophages may play an important role in maintaining NK activity in vivo, via their critical involvement in interferon prduction.

In addition to the studies related to macrophages, the effects on NK activity of a variety of immunopharmacologic treatments were examined. In both rats and mice, spontaneous levels of NK activity were shown to be inhibited by treatment with hydrocortisone, cyclophosmamide, or lethal X-irradiation. In contrast, these treatments had no detectable effect when administered prior to boosting with poly I:C. These findings led to the hypothesis that NK cells develop from precursors which are substantially more resistant to X-irradiation and cytotoxic drugs. The differentiation from pre-NK cells to functionally active NK cells appeared to be independent of proliferation, since treatments which inhibit DNA synthesis, including methotrexate, X-irradiation, and cytosine arabinoside had no effect on the ability of animals to be boosted.

These recent observations regarding the factors affecting NK activitiy in vivo as well as in vitro have provided important clues which should allow more definitive evaluation of the role of NK cells in in vivo resistance to tumor growth and in immunological surveillance. In collaboration with investigators at the University of Perugia, we have been correlating our findings regarding NK activity with in vivo radioresistant inhibition of tumor cell growth. The in vivo assays consist of measurement of both inhibition of tumor cell growth in various organs by in vivo inoculation of radiolabelled iododeoxyuridine, and of rapid and direct measurements of in vivo cytotoxicity by inoculation of prelabelled tumor cells. Inoculation of mice with pyran or poly I:C, which had been shown to boost NK activity, have also been shown to substantially increase in vivo resistance to tumors. Similarily, the administration of carrageenan and silica, which have been shown to interfere with NK activity and with boosting have been shown to inhibit in vivo resistance to tumors. Although these correlative results are encouraging, it remains to be determined how much of the effects are due to NK cells and how much can be attributed to direct anti-tumor effects of macrophages. It should be possible to dissociate these effects, since the kinetics of activation of NK cells and of macrophages vary by several days. Experiments are now in progress to carefully examine these issues.

It has been suggested that NK activity may be due to cooperation between K cells and natural antibodies, with resultant antibody dependent cell-mediated cytotoxicity. This possibility became quite important to consider when we found an impressive correlation between NK antivity and K cell activity in mice. An extensive series of experiments were performed to determine whether NK cells were actually K cells armed in vivo with natural antibodies via their Fc receptors. A variety of experimental approaches, including attempts to inhibit NK activity by Fab reagents directed against the Fab portion of IgG, have given essentially negative results. At this time, it seems unlikely that NK activity can be accounted for by any form of antibody dependent cell-mediated cytotoxicity.

II. Cellular Immune Reactions in Rats and Factors Affecting these Responses.

Studies of immunity of rats to the W/Fu Gross virus induced lymphoma, (C58NT)D, have continued. The principal focus during this past year has been on the production of migration inhibitory factor (MIF) in response to these tumor cells. This has been examined by the indirect microagarose droplet technique, using mouse peritoneal macrophages as the indicator cells. A major finding, which may be analogous to the natural cell-mediated cytotoxicity in rats, has been that normal W/Fu rats, particularly those greater than 6 weeks of age, have the ability to produce MIF upon incubation with the established tissue culture cell line of (C58NT)D. In an examination of the specificity of this phenomenon, thus far only rat tumor lines infected and transformed with Gross leukemia virus have elicited MIF production. A series of other tumor lines have been negative. As a possibile further analogy to rat NK reactivity, BN rats, which have low NK reactivity, have also been found to have little or no ability to produce MIF in response to these tumor cell lines. A further characteristic of this response has been the requirement for histocompatible macrophages, both in response to intact tumor cells as well as to 3M KCl extracts of tumor cells. The apparent requirement for histocompatible macrophages for MIF production in response to intact tumor cells has been somewhat surprising, since in similar studies in mice, macrophages were only required for responses to soluble tumor antigens.

Significance to Biomedical Research and the Program of the Institute:

Detection of tumor antigens and of immune reactions to tumor-associated antigens is essential for progress in tumor immunology. The sensitive assays of cell-mediated and humoral immunity which have been developed in this laboratory have already proved useful in studying the immune response to animal and human tumors.

The ability of our laboratory to perform multiple assays of cell-mediated immunity to tumor antigens, and to characterize the effector cells, should allow detailed dissection of the role of the immune responses in limiting or preventing tumor growth. These assays may also be useful in immunodiagnosis of cancer and in measuring changes in immunity, in response to chemotherapy or immunotherapy.

Natural cell-mediated immunity may play an important role in immune surveillance against tumors. Understanding of this phenomenon and demonstration of its in vivo significance in mice should be very useful for the understanding of the significance of human natural cell-mediated immunity, which seems to be mediated by the same or very similar types of effector cells.

Proposed Course of Project:

Studies on natural cell-mediated cytotoxicity in mice are being continued. A major emphasis will be on a detailed evaluation of the role of NK cells in in vivo resistance against tumor growth. We will make use of the recent information regarding the apparent role of macrophages and of interferon in regulating NK reactivity and our protocols will be focussed on attempts to discriminate between the anti-tumor effects of NK cells and of macrophages. Efforts will also be continued to better understand the factors regulating the spontaneous appearance and maintenance of NK levels in mice and to determine what the role of macrophages and interferon production are in the natural events. It will be of considerable interest to determine whether the variation in NK reactivity among different strains can be accounted for by the susceptibility to activation by interferon.

Studies on cellular immunity of rats to the (C58NT)D lymphoma will be continued. A particular emphasis will be on the nature of the relationship between natural cytotoxicity and the natural development of the ability to produce MIF in response to the tumor cells. The nature of the cells responsible for each of these phenomena will be carefully examined.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08500-04 LID

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunodiagnosis, Immunotherapy and Immunological Monitoring in Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.D. Bonnard	Visiting Scientist	LID NCI
	W.H. West	Senior Investigator	LID NCI
Others:	R.B. Herberman	Chief	LID NCI
	J.R. Ortaldo	Biologist	LID NCI
	H.D. Kay	IPA	LID NCI
	R.D. Maca	IPA	LID NCI
	J.M. Gerson	IPA	LID NCI
	N.P. Lang	IPA	LID NCI
	K. Yasaka	Guest Worker	LID NCI
	R. Fagnani	Guest Worker	LID NCI

COOPERATING UNITS (if any) Litton-Bionetics, Inc.; M, NCI; Surg., NCI; PO, NCI; Met, NCI; GW Univ., Georgetown Univ., D.C./VA Hosp.; Univ. Iowa; Univ. Penn., Univ. Ark., Scripps Clin.; Natl. Naval Med. Ctr.; Walter Reed Med. Ctr.; Portsmouth Naval Hosp; Univ. South Carolina; Kaiser Permanente Hosp.

LAB/BRANCH

Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10	8	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Characterization of natural cytotoxicity and natural killer cells. Comparison with lymphocytes that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against Chang liver cells. Study of the generation of natural cytotoxicity and ADCC in 5-7 day cultures. Enhancement of these activities by co-culture with antigens, mitogens, tumor cells, immunoadjuvants and interferon. Comparison of these activities with the generation of specific immune T lymphocytes in mixed lymphocyte cultures. Use of continued cultures of pure T lymphocytes from any given individual and characterization of their functional activities. Immunological monitoring of patients with lung and breast cancer, Ewing's sarcoma, and melanoma. Adjuvant chemotherapy and immunotherapy with BCG, C. parvum, and poly I:C. Skin tests with purified, soluble extracts of tumor cells. In vitro assessment of immunocompetence, natural cytotoxicity, and tumor immune reactivity, by short-term cytotoxicity assays, mixed lymphocyte tumor cell culture, and leukocyte migration inhibition. Reactivity of subpopulations of lymphocytes. Examination of cells from various lymphoid organs and from the tumor.

Project Description:

Objectives:

I. Studies on Cytotoxicity

We continued our thorough investigation on the specificity of diverse cytotoxic reactions *in vitro*, and on the effector cells involved. We define direct cytotoxicity as that measured in a 4 hr ^{51}Cr release assay (CRA) with fresh mononuclear peripheral blood leukocytes (MPBL) or their subpopulations. We tried to better characterize the effector cells of the spontaneous cytotoxicity or natural killing (NK), and to identify specifically immune cytotoxic lymphocytes (CTL), with reactivity against tumor-associated antigens (TAA). We discussed a role for natural antibodies and antibody-dependent cell-mediated cytotoxicity (ADCC) in NK. We examined the possible role of lymphotoxins (LT) in both ADCC and NK. We performed cell separations on the basis of cell-surface markers or size, and used the purified effector cells. We examined effector cells from various organs, and from cord blood.

We completed a similar investigation on the specificity of indirect cytotoxic reactions generated *in vitro*, and on the precursor cells and effector cells involved. We define indirect cytotoxicity as that assessed at the end of a culture period of 5-7 days, with MPBL cultured alone, or with various stimuli, including tumor cells. Cell separations were done at the onset and at the end of cultures, and the various resulting fractions were tested in NK and ADCC cytotoxicity. We started an in depth study on the role of interferon, and interferon-inducers, in boosting NK and ADCC activity, both *in vitro* and *in vivo*. We reestablished the mixed lymphocyte culture and cell-mediated lympholysis (MLC-CML) assay in our laboratory as a privileged model for the generation of specific immune CTL.

We tried to better understand the role of NK in tumor immunity and started to measure systematically this activity following courses of chemotherapy or immunotherapy. This is to attempt a parallel with animal studies that have demonstrated that NK has some importance for rejection of tumors, and that NK levels are considerably boosted by injections of various immunoadjuvants.

II. Cell Separation Studies and Generation of Pure T Cells

The usefulness of cytotoxicity experiments, and other assays of cell-mediated immunity, as correlates for *in vivo* tumor immunity and rejection, depends, in our opinion, largely on the precise identification of the cells involved in each step of the reaction. In addition, it is important to learn whether these cells are present in the tumor itself, or in the draining lymph nodes. Therefore, we have utilized a very systematic approach with separation of MPBL into various subpopulations on the basis of cell surface markers and differences in size. Then we tried to recover similar population of leukocyte populations from the tumor itself. We monitored closely the resulting fractions for morphology, cell-surface markers, and we tested them in functional assays.

We continued our sustained efforts with continued cultures of normal human T cells _in vitro_. We demonstrated that these cultured T cells (CTC) retained specific immunological memory, and several functions of normal, fresh T lymphocytes. We gained much insight into means to produce the conditioned media that allow the continued growth of the CTC. Because a very analogous system is available in the mouse, it will be soon possible to document the feasibility of performing immunological reconstitution and immunotherapy with these cells.

III. Immune Monitoring of Cancer Patients

A major effort of the Laboratory of Immunodiagnosis has been the immunological monitoring of cancer patients with _in vivo_ and _in vitro_ tests. Our studies involve collaborating institutions where patients receive primary care. Much data were analyzed, which were obtained serially during the course of lung and breast cancer, and of melanoma and Ewing's sarcoma. The aims of this part of the Laboratory were: 1) To design protocols for the immunological monitoring of cancer patients at the collaborating institutions. 2) To organize the logistics regarding these protocols, including regular patient follow-up, collection of blood samples and tumor specimens, and distribution into the proper tests as the testing laboratory. 3) To perform skin tests with recall antigens and purified tumor extracts, and to help in setting up priorities for the step by step purification of tumor-derived antigens. 4) To help evaluate and summarize data for publication.

Major Findings

I. Natural Reactivity and Tumor Immunity in Cytotoxicity Assays

A. Mechanisms of Natural Cytotoxicity and ADCC

Our work from the previous year, as well as studies from other laboratories, have left little doubt as to the identity of the NK cell. It is an FcR+ lymphocyte of the T cell lineage, inseparable from and perhaps identical with the killer (K) cell that mediates ADCC in the well-defined Chang liver cell system. This year we were able to confirm the T cell lineage of the NK and K cells by using a specific anti-T cell serum (see below). Treatment of effector cell populations with anti-T cell serum and complement resulted in abrogation of NK and K. This may not simply be due to formation of immune complexes, since the cells were thoroughly washed after treatment, and since not all complexes inhibit NK. The anti-T cell serum reacted not only with sE-RFC but also with a large proportion of the non-sE-RFC found in the interface after sheep erythrocyte rosette cell separations, that was also responsible for the NK activity of the interface.

The cytotoxicity tests were performed in a 4 hr CRA with fresh, normal MPBL: for NK activity against the K-562 myeloid cell line; for ADCC against rabbit antiserum sensitized Chang liver cells. We also used two breast cancer derived cell lines (MCF-7 and MDA-231), two lung carcinoma lines (CaLu and E-14), a Ewing's sarcoma line (5838), and a colon carcinoma line (HT-29) as additional target cells for tests of specificity.

In order to decide whether NK and K cells were actually the same, we initiated a series of experiments with adsorption of the effector cells on specific target-cell monolayers. Thus, NK cells would be adsorbed on NK target cells, and the non-adherent cells tested for (NK and) K cell activity. As further evidence for the identity of NK and K cells, we planned on blocking ADCC reactions with good NK target cells as opposed to getting little if any inhibition with target cells not susceptible to NK. We encountered several problems with these approaches, so that we have not yet settled this particular question: 1) K-562 target cells grow in suspension cultures, and our attempts to derive an adherent variant have been only partially successful. 2) K-562 have numerous cell surface FcR, which could possibly interfere directly with antiboby-coated target cells and hence with ADCC reactions. 3) E-14, a lung-cancer derived cell line growing in monolayer, was found to have excellent susceptibility to NK, similar to K-562, and had no FcR. However, we have not been able to consistently remove NK activity on E-14 monolayers, and therefore their effects on ADCC are not yet clear. The use of effector cells depleted of cells that are adherent to nylon-fibers may represent an improvement (Kiessling, personal communication). Whatever the results of these monolayer experiments may be, we already have evidence from cold target cell inhibitions of the CRA that NK and K cells are the same. It was possible to inhibit ADCC reactions with NK target cells, like E-14 and K-562, but not with target cells resistant to NK, i.e. the Chang liver cells. However, these experiments also demonstrated that ADCC reactions were considerably more difficult to inhibit than the NK reactions run in parallel. The result is quite compatible with the hypothesis we have recently made on the mechanisms of NK and ADCC, which is explained in the following two paragraphs.

Whereas most investigators agree on the similarities between the NK and K cells, there is much controversy as to whether NK activity involves the FcR and IgG antibodies. During the period under review we have accumulated several pieces of evidence showing that IgG antibodies attached to FcR play no role in NK: 1) Although one laboratory has claimed that NK was inhibited by monovalent fragments (Fab fragments) of goat and rabbit anti-human IgG, we have not been able to reproduce these results with an excellent monovalent Fab fragment of goat anti-F(ab')2 reagent. 2) Although a different laboratory has claimed that NK was due to K cells armed with natural IgG antibodies, as shown by specificity testing and rearming of trypsinized effector cells, we were unable to reproduce these findings, even after an extensive series of attempts under various conditions. 3) We have established that several immune complexes that completely block ADCC do not block NK. This is difficult to explain if NK were due to IgG antibodies on the FcR. 4) Also, most investigators who have dealt with FcR cytophilic IgG antibodies have described their binding as weak, and very labile at 37°C. In contrast, we showed that NK is quite stable at 37°C for several hours. 5) In addition, our studies on the generation of NK in vitro suggest that B cells and/or antibody production are not needed to generate NK activity (see below).

We have recently proposed an alternate model which accounts for the identity of NK and K cell effector mechanism and yet ascribes no role to the FcR and IgG antibodies during NK. Carefully based on data available in the literature, this model proposes that lymphotoxin (LT) could well be the mediator used for lysis by NK and K cells. Whereas NK target cells would bear a receptor for and be very susceptible to a form of LT expressed on the effector cell surface, target cells used in ADCC may be quite resistant to this baseline spontaneous activity. The model proposes that NK and K cells are physically the same, and that an NK reaction becomes an ADCC reaction as soon as IgG antibodies start to coat the target cells. The main effect of the reaction of antibody-coated target cells with the FcR on the effector cell is not the "binding" between the two cells as previously thought, but rather a metabolic activation of the cell via the FcR that turns on production of more LT, or of a more active form of LT. We have initiated a collaboration with the group of Dr. Gale Granger working on LT to try to confirm this hypothesis. 1) It should be possible to inhibit NK and ADCC with well-characterized anti-LT sera. 2) It should be possible to detect LT at the surface of effector cells, and particularly at the surface of those FcR+ cells that mediate NK and ADCC. Our preliminary results are at least consistent with these expectations.

B. Generation of NK and ADCC Cells In Vitro

Interesting data were obtained again with our model of in vitro generation of NK and K cells. MPBL cultured alone in fetal bovine serum maintain good levels of NK and ADCC. This is much enhanced by the presence of x-irradiated K-562 cells. If MPBL are first depleted of FcR+ cells, to deplete their NK and ADCC, these activities reappear in cultures with fetal bovine serum, or with K-562, within 5-7 days. During the period under review, we particularly analyzed the cellular requirements for the regeneration of these activities. Sheep erythrocyte fractionation studies revealed that neither the E-RFC pellet nor the non-E-RFC interface were able to regenerate much activity. However, when both E-RFC and non-E-RFC were mixed together good regeneration occured. It was possible to generate activity after x-irradiation of the E-RFC fraction but not when the non-E-RFC were irradiated. Within this latter fraction, the null cells, rather then the B cells, were found to be required for generation of NK and K cells.

Our earlier observation that K-562 was very effective in inducing NK and K cells, received a possible explanation from the observation of Trinchieri that NK was boosted by interferon in these cultures. We had also observed the effect of interferon and interferon-inducers in animal systems, both in vivo and in vitro. It was therefore of interest to look at the effect of interferon in our in vitro system for human MPBL. We found that purified interferon preparations from lymphoblastoid cell lines, fibroblasts, or lymphocytes were all able to boost preexisting NK and K cell activities. However, interferon did not appear to facilitate the development of FcR-precursor cells into FcR+ effector cells. Experiments using interferon-inducers and specific anti-interferon antibodies are in progress. Also, we have now the possibility to monitor patients receiving poly I:C-polylysine complex, for their levels of NK and ADCC activities.

C. Specificity of Cytotoxic Reactions

In our previous report we expressed some hope that we could possibly distinguish and separate NK activity due to FcR+ cells, from disease-related cytotoxicity due to FcR- cells. We have gained considerable experience with this during the present year, and have improved our means of assessing the results of cytotoxicity. We are now less optimistic and realize that: 1) FCR- cell cytotoxicity against allogeneic tumor cell lines was not detected frequently. 2) It did not appear to be disease-related but could appear in normal, healthy donors, as well as in cancer patients. However, these infrequent effects could well be due to specific immune cytotoxic T lymphocyte activity.

These conclusions are based on our screening of breast cancer patients, benign breast disease patients and normal individuals for disproportionately high reactivity against MCF-7 and MDA-231 and relatively low reactivity with K-562 or in ADCC. We improved the measurements by introducing higher ratios of effector to target cells against the two breast cancer-derived cell lines, and by expressing the results in lytic units rather than in percent of lysis at one particular ratio. The three groups of patients did not differ with respect to the frequency of individuals who reacted particularly strongly to MCF-7 and MDA-231. This is in contrast to a previous report from our group, and the reasons for this are unknown, but may be related to the recent use of standard preparation of cryopreserved MCF-7 cells, which may not have been sufficiently antigenic. When those individuals that reacted strongly to MCF-7 or MDA-231 were recalled, they usually reacted strongly again. We then carried out immunoadsorption experiments on EA monolayers, and cytotoxicity was then tested using the non-adherent fraction of lymphocytes. This procedure almost completely eliminated cytotoxicity against K-562, and also against the other cell lines. However, very few individuals were identified whose activity against MCF-7 and MDA-231 was due to FcR- cells, rather than to FcR+ cells. We then embarked on cold target cell inhibition studies in the CRA to determine the specificity of the killing mediated by such FcR- cells. These tests which could be performed only on two individuals so far, seemed to indicate the recognition of a common antigen on MCF-7 and MDA-231, with less reactivity with the other cell lines tested (CaLu, a lung carcinoma line, and 5838, a Ewing's sarcoma line).

Because of the extent of our effort with EA-monolayer adsorption, we have not pursued adsorptions on monolayers of target-cells this year, except as indicated above in attempts to remove K cells.

The specificity of the NK activity regenerated after 7 day culture of MPBL was also examined and compared to that of the same fresh, normal MPBL tested on day 0. This was again done by cold target cell inhibition in the

CRA. In most instances, the specificity after regeneration on day 7 was very similar with that observed with the MPBL on day 0. This shows that the activity on day 7 of culture is that of NK cells, and strongly supports our contention that our culture system represents an in vitro model for the generation of this activity.

II. Cell Separation Studies and Generation of Pure T Cells

A. Cell Separations and Monitoring of Cell Surface Markers

Good techniques for cell separations and close monitoring of all fractions by means of adequate cell surface markers represent one of the practical goals in our laboratory. More sophistication was achieved this year by the introduction of F(ab')2 anti-human Ig columns in our work on the generation of NK, with the kind help of Dr. R. McDermott. Further, we introduced the use of specific anti-T cell and anti-B cell sera, by courtesy of Drs. Kaplan, Metzgar and Revillard and also following the development of our own anti-T cell serum (see below). These reagents were particularly useful to characterize the NK cells as belonging to the T cell lineage.

B. Separation of the Cellular Components of Human Tumors

Almost all studies of cell-mediated immunity in cancer patients have been performed with MPBL. However, cells within the tumor themselves may be more relevant to responses to tumors, particularly in regard to resistance against tumor growth. Therefore, we continued our study to separate and examine the various components of human tumors. The 1 g velocity sedimentation and isokinetic gradient fractionation techniques used the previous year proved too complicated for handling tumor specimens at this early stage of the study. Simpler procedures are being developed, particularly a discontinuous sucrose gradient that seems promising. Recovery was excellent, and viability often quite good. It was evident that numerous lymphoid cells could be recovered from tumors such as lung cancer, colon cancer and neuroblastoma. They could then be tested for various lymphocyte functions.

Human neuroblastoma and lung and colon cancers were studied. The infiltrating lymphoid cells in the tumors have been heterogeneous with macrophages being more numerous than lymphocytes. In all instances there was no detectable NK or ADCC activity in lymphocytes, obtained from tumors, whereas the patients' peripheral blood had activity. Studies are in progress to further define the effector cell populations present and to determine why cytotoxic cells cannot be recovered from the tumor.

C. Generation and Continued Growth of Pure T Cells

1. Conditions for the Generation of the Needed Mitogenic Factor: Human Cultured T cells (CTC) will grow for 3-4 weeks when in the presence of 10-20% of a 4-fold concentrated, commercially available conditioned medium from pooled, allogeneic, PHA-stimulated MPBL. CTC possess many of the typical characteristics of isolated T lymphocytes. CTC will allow crucial studies of

the functional and biochemical properties of human T cells, that are uncontaminated with other MPBL. Obtaining such large numbers of cells is extremely dependent on very effective conditioned media, which at present are not routinely available. We have studied the conditions required for production of the mitogenic factor (MF) in conditioned media, by MPBL from single donors, using PHA or Con A, and various concentrations of pooled human serum. (Various preparations of conditioned media were assayed in serial dilutions by 3 day long stimulation of 3rd passage, 14 day old CTC (10^5) in microtiter wells, followed by a 4 hr ^{3}HTdR pulse.) MPBL were often obtained by leukophoresis of normal volunteers. The best concentration of MPBL for producing conditioned media was 10^6/ml. There was often good MF activity in cultures with 1μg/ml PHA, or 40μg/ml Con A, with or without serum. However, the variation in MF content of conditioned media harvested at different times was so great that a pattern emerged only after several experiments. With 1% and 5% serum, the results were 24 hr > 48 hr > 14 hr > 72 hr > 6 hr. With 0% serum, 48 hr was often optimal. MF activity disappeared shortly after initiation of proliferation in the PHA-stimulated cultures, and this suggests a feed-back regulatory mechanism. Preliminary experiments allowing for better and more sustained production of MF in the presence of 10^{-6}M indomethacin or after removal of monocytes support the possibility that prostaglandins from monocytes may be the regulatory mechanism. Also, PHA-transformed blasts in these cultures readily remove the MF activity from the conditioned media. This phenomenon was avoided by 2000 R X-irradiation of the responding MPBL: production of MF occured normally and the cells did not remove activity from the conditioned media. By continuing these various improvements, we hope to overcome what have been serious problems with the production of conditioned media during the period under review.

2. Functional Characteristics of the Mitogenic Factor Used in Growing CTC: MF was shown to be stable at -70°C or 4°C for months, and at 37°C for at least 7 days. Small volumes of conditioned medium (0.3ml) incubated with 2-10 x 10^7 CTC lost significant activity within 1 hr. Although attempts to elute MF have been unsuccessful so far, we think that absorption through a cell-surface or cytoplasmic receptor could have taken place. As indicated above, 72 hr PHA-(or Con A-)blasts removed considerable MF activity, whereas MPBL or HSB cells (a T cell line) did not. These data suggest a very interesting two-stage model of MPBL activation by mitogenic lectins: First, lectins activate MPBL to produce the MF. This is an early phenomenon, which does not involve DNA synthesis and which is radioresistant. Second, a very minor population of MF-receptor positive T cells in MPBL, or perhaps only some T cells that have acquired a MF-receptor after contact with the lectin absorb the MF and enter mitogenesis. Our data indicate that the full development of a sizeable MF-receptor positive population is a radiosensitive phenomenon. The data are consistant with the two stages of lectin-induced proliferation occuring in the same T cells, or in different subsets of T cells. However, we have found that the CTC themselves have eventually lost the ability to proliferate in presence of mitogenic doses of lectins. This speaks strongly in favor of the second possibility, i.e. that the MF-receptor positive T cells and CTC represent a subpopulation of T cells. It will now be important to determine the subpopulation of MPBL which produce MF, as opposed to the sub-

populations which respond to it and eventually become CTC.

3. Allogeneic Reactivity of the CTC: Mixed lymphocyte cultures (MLC), using 10-21 day old CTC as responders and allogeneic irradiated MPBL as stimulators, were set up to measure ^{3}HTdR incorporation, from day 1 through day 7. CTC consistently showed an early (day 2-4) ^{3}HTdR incorporation reaction with allogeneic MPBL. They did not react with autologous MPBL or CTC. An HLA typed family showed that CTC reactivity was determined by antigens coded for by the major histocompatibility complex. Using homozygous MLC-typing cells as additional X-irradiated MPBL, CTC were shown to react only when these X-irradiated MPBL were different with regard to HLA-D region antigens. However, we have reasons to believe that the CTC did not respond directly to HLA-D region alloantigens. Rather, with HLA-D region antigens they may stimulate the production of blastogenic factor by the X-irradiated MPBL. The CTC would then proliferate in response to such blastogenic or mitogenic factor. This hypothesis is being currently investigated. We are also attempting to develop CTC selectively reactive with only one HLA-D determinant.

The early kinetics of the CTC MLC responses is similar to the secondary type reactions seen in the primed lymphocyte test, and could possibly be due to exposure of the CTC to soluble alloantigens present in the conditioned media made from 10-20 allogeneic donors. However, we were able to grow CTC in conditioned medium prepared from one donor, the same as the donor of the CTC. These CTC showed the same early kinetics of MLC responses. An alternate explanation for this secondary type reaction in MLC is the possible response to mitogenic factor, and not to alloantigens in these cultures.

CTC most often proved to be quite cytotoxic against a wide variety of tumor target cells. This cytotoxicity was often much enhanced by the addition of lectins, PHA or Con A, in the CRA. With many, but not all, conditioned media, the CTC also readily killed allogeneic PHA-blasts. They never killed autologous PHA-blasts. The specificity of this reaction was shown to be similar to that of MPBL from the same donor activated by a pool of irradiated MPBL: killing of all CML-histoincompatible PHA-blasts (as defined in the regular MLC-CML) and no killing of the CML compatible or of autologous PHA-blasts. This may become very useful for prospective, rapid studies of CML histocompatibility in transplantation. That CTC became polyclonal activated cytotoxic cells with some, but not all conditioned media, is of considerable interest and warrants further studies. We have recently been able to show that polyclonal activation can also take place in autologous conditioned media. prepared from MPBL of the CTC donor. Thus, polyclonal activation must be a consequence of exposure to the mitogenic factor (and the PHA?), and not to soluble alloantigens. We hope to learn soon whether a particular subset of T cells respond in this manner, and we expect to derive useful information for our general understanding of subsets that mediate cytotoxic reactions *in vitro*.

4. Suppressor Activity of the CTC: Extensive studies have been performed, which demonstrate that CTC act as suppressor cells in at least

three different systems: antibody production in vitro, proliferative responses to Con A, and proliferative responses in MLC. Although these activities need to be further characterized, and the responsible T cell subsets isolated, this again points towards the CTC being an interesting source of functional subsets of T cells. Availability of suppressor T cells in large numbers may foster our understanding of suppressor mechanisms in cancer patients as well.

III. Immune Monitoring of Cancer Patients

A. In Vitro Delayed Hypersensitivity Testing of Tumor Immunity

We continued to measure delayed hypersensitivity of patients to Sephadex G-200 and polyacrylamide gel electrophoresis (PAGE) purified soluble fractions of tumor cell extracts, prepared by Dr. A. Hollinshead. The analysis of the large scale testing with large batches of purified extracts prepared from standardized sources (mainly tissue culture cell lines shown to be antigenic in in vitro assays), showed that the results did not correlate well with the course of the disease. Thus, the major emphasis during the past year has been to test selected cancer patients, with various fractions from a given antigen-separation. This was being done to help evaluate the fractions containing tumor associated antigens. PAGE fractions from Ewing's sarcoma line 5838, were tested and were found non-reactive at 5μg/0.1ml. Positive reactions were observed with several PAGE reactions from a large metastasis of melanoma. Not enough material was available to ascertain that this was disease-specific.

B. Cellular Immune Competence and Suppressor Cells

Patients with lung and breast cancer, who showed evidence of depressed proliferative responses to PHA, Con A and in MLC were systematically recalled, and their MPBL were analyzed for evidence of suppressor cells. In analogy with work done in mice and rats in our laboratory, MPBL were passed over G-10 Sephadex columns to remove monocytes. Under these circumstances the lymphocyte proliferative responses often returned to normal values in lung cancer patients, suggesting an adherent suppressor cell. Indeed, unseparated MPBL or cells adherent to the G-10 columns were able to suppress the responses of normal individuals to PHA, Con A and in MLC. In cancer of the breast, some patients had suppressor cells that were not removed by the G-10 columns. Whereas both types of suppressor cells appeared to be present more often in patients with extensive disease, it is difficult at this stage to make precise clinical correlations.

C. 29°C and 4°C Rosette Assay

Our previous studies with the 29° rosette assay in lung cancer patients suggested that it might provide useful prognostic information. We have therefore performed a detailed analysis on the Stage I lung cancer patients on the randomized immunotherapy protocol. Rosette levels were determined on most patients on a monthly basis and the data were analyzed to determine whether the proportion of depressed rosette values during the first six months after surgery could predict subsequent clinical course. Among patients with 1/3 or more of their tests depressed during this period, a higher proportion relapsed than did those patients who had a lower incidence of depressed tests and this difference is approaching statistical significance. These results have suggested that a repeated rosette testing on lung cancer patients during the first few months after surgery might help to more accurately stage the patients.

When the results of the first test performed on Stage I lung cancer patients in the rosette assays were combined with the results of lymphoproliferative responses to PHA and MLC, the results from this single time point provided essentially equivalent discrimination to that obtained by sequential testing by either the 29° rosette assay or the lymphoproliferation assay.

In addition to the studies on Stage I lung cancer patients, this assay was also performed on a large number of samples from patients with other types of cancer. As before, a substantial proportion of patients with breast cancer and malignant melanoma were shown to have depressed proportions of 29° rosette forming cells in comparison to normal controls.

From previous studies, it appeared that the proportion of high affinity rosette forming cells and not the total proportion of T cells were depressed in many cancer patients. These data suggested that there was a shift in the subpopulations of T cells rather than an absolute depression in the number of T cells. Since low affinity rosette forming cells correlated closely with the T_γ, it seemed quite possible that depressed proportions of high affinity rosette forming cells would correlate with depressed proportions of T_μ. Therefore, a number of PBL preparations from cancer patients were compared to those of normal adults for the proportion of T_μ cells. Most of the cancer patients studies had decreased proportion of T_μ cells, and studies are currently performed to determine whether this measurement may be as useful or more useful than the 29° rosette assay.

D. Responses of MPBL to Autologous Tumor Cell Extracts

Lung and breast cancer patients were tested for the proliferative responses of their MPBL to autologous tumor material. We could confirm that 60% of our tests in lung cancinoma and 40% of our tests in breast carcinoma resulted in proliferation. Low concentrations of hypotonic membrane preparations worked particularly well in this system. It could be demonstrated that LIF is also produced in these cultures. Several patients were recalled for these studies. We have suspended experiments which showed the development of cytotoxicity in these cultures, because of our increased awareness of non-specific reactions which complicate interpretation of results. There is however a good prospect to detect specifically immune cytotoxic lymphocytes in those autologous situations where the target cells and the effector cells come from the same patient. We therefore plan to use fresh tumor cells and tumor cells that have been grown in culture as stimulators and target cells, and to test cryopreserved and and/or fresh effector lymphocytes from the same patients for their proliferative and cytotoxic responses and for their production of lymphokines in response to autologous tumor antigens.

E. Leukocyte Migration Inhibition

See report Z01 CB 08522-02

F. Previous Concepts and New Directions of our Clinical Protocols

Much of the testing indicated above was done with the aim of serially monitoring cancer patients, on a monthly or bi-monthly basis, for their baseline general immune competence or specific antitumor immunity. For this reason, tests were done as far away as possible from therapy, i.e. usually at the end of each cycle of therapy, just prior to the next cycle. It has been generally assumed that tests performed under these circumstances may reflect the immune status of the patient, and may correlate with the extent and course of the disease. The 29° rosette assay and lymphoproliferative responses to PHA and MLC have yielded data which fit this expectation, depressed values being associated with poorer prognosis and shorter disease-free intervals. LMI reactivity against disease-related antigens seemed greater when the tumor load was greater, i.e. prior to operation, or after recurrence. LMI reactivity often remained until death occured. The information gained from our serial testing was sufficient to warrant continuation of these tests in selected clinical situations. We have concentrated on the longitudinal testing in LMI of one series of breast cancer patients with very purified materials, which may allow characterization of tumor associated antigens in some of the fractions, (see report Z01 CB 08522-02), and similarly on one series of lung cancer patients, who are also tested in the 29°C and 4°C rosette assays.

The serial monitoring of cancer patients has failed to reveal any modification of the tests in response to immunotherapy with either BCG or cells. This may be related to the timing of the assays, which were all done at a point in time as distant as possible from the therapy, i.e. just before the next cycle. The failure of our tests to reflect any effects of the non-specific or specific immunotherapy is similar to observations made in many other laboratories. In order to optimize protocols for immunotherapy it would be helpful to have _in vitro_ assays which reflect important immunological responses to the therapy. Much thought was given to newer concepts of immune monitoring. (1) The effects of non-specific immunotherapy with agents such as BCG or _Corynebacterium parvum_ are not well understood. Considerable evidence obtained very recently in animal studies shows that immunotherapy with a variety of microorganisms and material had profound, and hitherto unknown, rapid effects on functions such as natural killer cell and killer cell activity, and on cytostasis mediated by macrophages. Most recent evidence from this laboratory, and from other laboratories as well, ascribes the effects on NK and K cells to interferon, which is always induced by these immunotherapeutic regimens. Because these biological activities are now thought to be extremely important in immune surveillance and tumor immunity, and because the in vitro tests for these functions are already available in man and reproducible, it was decided to also look in humans at the rapid enhancing effects of immunoadjuvants on NK, ADCC, and monocyte-mediated cytostasis. In addition, it was decided to continue to determine the proportion of lymphocytes forming rosettes at 29°C and 4°C as a good indication of subpopulation of T cells, one of which (4°C rosettes) mediates NK and ADCC. We would also store 5ml of serum at each point in time in these protocols. (2) In the case of injections of autologous and allogeneic tumor cells, it is important to detect a rise in the patient's reactivity to these materials, in particular to tumor associated antigens. Because no boosting of specific anti-tumor reactivity had been seen late after treatment, we decided to measure it earlier after injection of these materials. Particularly, boosting of reactivity after injection of autologous tumor materials may indicate reactivity acquired against tumor-associated antigens. The tests that were chosen to measure reactions to specific materials are the LMI assay, and the mixed lymphocyte tumor cell interaction. (3) It is important to start with a good baseline value for the test, which we determine on 2 or 3 samples of blood collected prior to therapy. Because of the early kinetics of the effects seen in animals, we then take 3 further blood samples during the first week post-therapy, and then 1 sample every week up to a month. Each patient need not participate in these studies more than 1 or 2 cycles of therapy. Our preliminary results with this approach suggests that we do in fact see variations in the parameters measured. Analysis of these results is going on presently.

G. Clinical and Therapeutic Protocols

1. Protocols on Cancer of the Lung: We have been assessing the posible benefits of post-operative adjuvant immunotherapy with BCG and allogeneic tumor-cells, or BCG alone in a randomized controlled trial for stage $T_1 N_0 M_0$ patients. Preliminary results indicate a significant benefit of the BCG immunotherapy both in terms of disease-free interval and survival. The addition of allogeneic x-irradiated tumor cells had no influence on the results. Thus, the favorable results with $T_1 N_0 M_0$ using BCG are in contrast with our earlier observations of no effect on patients with more advanced disease. This is much in line with the concept that immunotherapy can only be effective when the bulk of the tumor has been removed. We are continuing accrual of $T_1 N_0 M_0$ patients on this protocol.

2. Protocol on Cancer of the Breast: The study on adjuvant chemo-immunotherapy in cancer of the breast has been discontinued by Medicine Branch, NCI. We have been able to monitor further those patients already on protocol, who have not recurred. Stage II patients had been randomized to (1) L-PAM, (2) Cytoxan, Methotrexate, and Fluorouracil (CMF), and (3) CMF plus immunotherapy with *Corynobacterium parvum*. The analysis of our serial data obtained with these patients in stimulation assays, LMI and rosettes, fails to reveal a good correlation with the clinical course of the disease.

3. Protocol on Ewing's Sarcoma: Patients on adjuvant chemotherapy following irradiation of their primary tumor, and patients on chemo-

therapy for metastases to other sites were followed-up. It is too early to analyze the possible benefits of these treatments. The patients were serially tested in LMI with a KCl extract of a Ewing's sarcoma line: 5838, but the outcome of these tests failed to correlate well with the course of the disease. One set of more purified PAGE preparations was found non-reactive. LMI testing has been discontinued. A pilot randomized study using immunotherapy with BCG and allogeneic tumor cells on patients with metastases, and with "high-risk" primary lesions of the trunk and proximal segments of limbs did not show any dramatic differences, and was discontinued in favor of the more aggressive chemo- and radiation therapy protocols. The immune monitoring of all these patients has been redirected as indicated above to monitor closely the immediate effects of intense courses of chemotherapy, and - in selected patients - of whole body irradiation.

4. Protocols on Melanoma Patients: Our clinical collaborator, Dr. Max Cohen, has left NIH, but still keeps clinical responsibility for patients as a consultant in the Clinics. Patients with locally recurrent lessons were randomized to (1) local injection of BCG, and (2) local injection of DNCB. The emphasis of immune monitoring was on simultaneous testing of PAGE purified melanoma extracts in skin testing and in LMI on a bimonthly basis. We are also interested in monitoring closely the patients of Dr. Cohen who receive phase two immunotherapeutic injections of PAGE purified melanoma extracts in complete Freund's adjuvant. As indicated above, this testing involves measurements of NK, ADCC, cytostatic activity of macrophages, 4°C and 29°C rosettes and LMI.

Personnel and Collaborators

We were very unfortunate during this year to lose a fine collaborator and scientist. Arlene Levin, who had been in our laboratory since 1976 died of metastatic breast cancer only a few days after she had to discontinue the pursuit of her excellent career as M.D. and scientist, due to complications of her disease. Arlene Levin had initiated with much success those experiments in our laboratory which are concerned with the separation of lymphoid cell populations from human tumors. She remains in our memories as an example of courage and dedication to her work.

Several collaborators visited this laboratory for limited times during the period under review. Ricardo Bernales, M.D., from Georgetown University, D.C., has studied subpopulations of lymphocytes in cord blood. Dolores Schendel, Ph.D. from the Sloan Kettering Institute in New York has worked on the generation of allospecific cytotoxic effector cells and their propagation in vitro with the cultured T cell technique. Mr. Carl Ware, from the University of California at Irvine, worked on the possible role of lymphotoxin in NK. Doris A. Morgan, Ph.D., worked on the biochemistry of the mitogenic factor that allows growth of the cultured T cells. Alberto Mantovani, M.D. from the Instituto Mario Negri in Milan, developed assays of human monocyte functions. Manuel O. de Landazuri, M.D., developed and used a technique for the selective absorption of lymphocytes on tumor cells, Jose M. Alvarez, M.D. and John S. Silva, M.D. worked with the mitogenic factor and the cultured T cells. These two investigators were from the University of Madrid. In addition, several students contributed to our studies.

Significance for Biomedical Research and the Program of the Institute

The capability to distinguish between NK activity and activity of immune CTL, that we developed in the laboratory, represents very significant progress towards the identification of CTL immune to TAA in cancer patients, a step generally viewed as crucial for the better understanding of CTL-mediated reactions that may be important *in vivo*. In addition, based on our data in animal systems, we feel that NK cells could also play a major role in immune surveillance and rejection of tumors. Their precise characterization, and the analysis of their specificity may represent the best way to investigate this further. The findings of our laboratory that NK as well as K cell activity can be boosted by interferon, which also seems to play a major role in the regulation of the spontaneous level of NK open major avenues of research aimed at better characterizing the regulation and the cellular requirements of this activity. The hypothesis that lymphotoxin may play a role in the actual killing of NK and K target cells is a new concept and related experiments may shed some light on the mechanism of cytotoxicity. This would fall under Objective V, Approach 4 of the National Cancer Plan.

The capability to generate large numbers of pure and functional T cells from any given individual opens considerable avenues for use of these cells in *in vitro* assays of cell-mediated immunity. Research will now have to be started on the potential use of these cells for immunotherapy. The technique may turn out to be the cleanest approach to the purification of considerable numbers of immunoreactive T cells, and it will certainly hold a very high priority in many cancer research laboratories for the years to come. The more general ability of our laboratory to fractionate subpopulations of lymphocytes, and of the cellular components of tumors, should allow detailed dissection of the role of the immune response in limiting or preventing tumor growth. These studies are also potentially useful for the immunodiagnosis of cancer and in measuring changes of immunity in response to chemotherapy or immunotherapy. This would fall under Objective V, Approach 4 and Objective VI, Approaches 1 and 4 of the National Cancer Plan.

The available techniques for the solubilization and isolation of tumor-associated cell surface antigens are important in their application to immunological monitoring, as described in this report. Such antigens may perhaps also be useful for therapy in the future. This would fall under Objective VI, Approaches 1, 2 and 4 of the National Cancer Plan.

Detection of tumor antigens and of immune reactions to human tumor-associated antigens is a major goal in tumor immunology. The development and standardization of *in vitro* assays for cell-mediated immunity is one of the most important aspects. Because immunogenicity cannot be studied directly in the human, the evaluation of immunologic reactivity and the antigenicity of tumor cells by those assays is of paramount importance. This would fall under Objective V, Approach 4 of the National Cancer Plan.

The question of whether immunosuppressive factors play a role in interfering with effective immunity is also very important. It appears that the stimulation of the host immune system may be useless, unless one can first rid the body of the inhibitory phenomena related to tumor cell growth. Investigation in this area would fall under Objective IV, Approach 4 and Objective VI. Approaches 1, 2, and 4 of the National Cancer Plan.

The use of various forms of immunotherapy in man has elicited considerable interest. It is imperative that the efficacy of such treatments be evaluated by clinical follow-up, and that the mode of action on the immunological system, if any, and the role of the immunological system during such therapy be clarified. This would fall under Objective V, Approach 4 and Objective VI, Approaches 1, 2, and 4 of the National Cancer Plan.

Proposed Course of Project

This project represents our laboratory's commitment to understanding the role of the immunological response of cancer patients to the tumor and tumor progression. As has been emphasized previously in this report, the functional integrity of each cell type in the immune system, as demonstrated by intact cell-mediated and humoral natural and immune reactions, as well as the capacity of a particular cell type to mount an effective response to the tumor, may be of paramount importance and should represent one of the ultimate goals of our research.

The priorities concerning our work with the cultured T cells will be as follows: (1) Production of the conditioned media in sufficiently large quantities; (2) Characterization of the mitogenic factor; (3) Generation of T cells with specific functions (responses to antigens and memory, helper T cells, suppressor T cells); (4) Cloning of T cells with specific function; (5) Histocompatibility reagents; (6) Antisera against T cells and T cell subsets; (7) T cell surface markers, idiotypes; (8) Mouse and nude mouse models; (9) CTC from cancer patients blood and tumor site.

The priorities concerning our analysis of the mechanism of NK and ADCC will be: (1) Demonstration of the physical identity of NK and K cells in human peripheral blood; (2) Demonstration of the role of interferon in regulating levels of NK and K cell activity, and analysis of its mechanism of action; (3) Further analysis of the specificity of NK cells by specific target cell monolayer experiments; (4) Analysis of a possible role for lymphotoxin in cytotoxic reactions; (5) Physical separation of NK cells and immune T cells and their precursors; (6) Relationship of NK to monocyte or macrophage mediated cytotoxicity.

The priorities concerning our immune monitoring of patients will be: (1) Selected studies of serial immunological and clinical data, and the development of adequate statistical methods for analysis of serial data; (2) Detailed immunological studies of selected patients undergoing immunotherapy with BCG, C. parvum or interferon-inducers such as Poly I:C Polylysine complex; (3) Performance of simultaneous *in vivo* skin testing, and *in vitro* LMI assays to examine the extent of correlation.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08511-03 LID

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Characterization and Measurement of Circulating Antigens Associated with Neoplastic Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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LAB/BRANCH

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.00	4.00	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of alpha-fetoprotein (AFP) and human chorionic gonadotropin (HCG) quantitated by radioimmunoassay (RIA) have been continued in clinical collaborations to aid in the diagnosis and monitoring of patients with hepatocellular carcinoma (HC) and germ cell tumors of the testis. Animal models for HC are available in monkeys and mice and are being studied for correlations of tumor size with serum AFP levels during tumor development and during various types of therapy. Development of a more sensitive RIA for AFP has allowed more accurate evaluation of serum AFP under normal conditions as well as in non-neoplastic liver disease. A RIA for a new human lung tumor associated antigen (LTAA) has been developed and used to demonstrate serum levels in lung cancer higher than those of normal controls. Studies are in progress to correlate the LTAA with stage of disease and evaluate specificity for differentiating lung cancer from benign lung disease and other types of tumors. A cross-reactive, but different protein has been discovered in normal human serum; isolation and identification of this antigen should lead to increased specificity of the RIA for LTAA. A non-virion surface antigen has been identified on a Moloney virus-induced lymphoma (MBL-2) and efforts to isolate and characterize this new antigen are in progress.

Project Description:

I. Characterization and Measurement of Soluble Antigens Associated with Human Neoplasms. Objective 6, Approach 1.

Studies of Alpha-Fetoprotein (AFP)

1. Quantitation of AFP in human disease: We have developed a more sensitive radioimmunoassay (RIA) for AFP which has reduced the background so that normal serum levels can clearly be measured. This has been accomplished by using a new antiserum with higher binding titer and by the addition of normal human serum, from which AFP has been specifically removed, to each reaction tube for the standard curve. With the more sensitive assay the normal serum AFP level was 3.64 ± 2.58 ng/ml for 321 subjects between 16-64 years of age. It also appeared that the serum AFP level may increase with age and may be higher in Blacks than in Caucasians. This work is being extended to determine the level of significance.

This same RIA for AFP has been used to determine quantitative levels of serum AFP in patients with hepatocellular carcinoma. Collaborations with several institutions in this country and abroad are aimed at the monitoring of therapy in these patients. Patients with elevated levels of serum AFP who demonstrated a response to therapy had a concomitant fall in AFP and this was generally a more sensitive and more rapid indication of response than were the usual clinical parameters. Furthermore, the AFP level usually provided the first evidence of resistance to the chemotherapy or to recurrent tumor growth.

The combined use of AFP and human chorionic gonadotropin (HCG) serum concentration has been demonstrated by our laboratory, as well as by others, to be an accurate and sensitive way to monitor the effectiveness of therapy in non-seminomatous germ cell tumors. Studies are underway at NIH and several other institutions to further define the sensitivity of these serum markers for predicting response to therapy and monitoring the remission period for detection of the earliest evidence of recurrent tumor. These same studies will provide further evidence for the value of these markers in staging of the patients and in providing prognostic aid. It is important that a single determination of markers not be overinterpreted and that serial values be utilized to interpret the post-operative course so that comparison of actual marker decline can be made with the expected decline consistent with the known catabolic half-life of the marker.

A survey of 60 patients with the diagnosis of pure seminoma revealed that 5 had elevations of serum HCG and 1 had elevated serum AFP. Elevated AFP or HCG in patients with seminoma could be the most sensitive indicator of a mixed tumor which should be considered for lymph node dissection and chemotherapy in addition to x-ray therapy.

An advantage of the more sensitive RIA is the ability to more accurately follow changes in serum AFP which are close to the normal level.

This has allowed the monitoring of patients with non-malignant liver disease, to determine the clinical correlations with serum AFP in acute infectious hepatitis and in chronic cirrhosis.

In patients with chronic liver disease the serum AFP levels remained in the normal range despite the appearance of dysfibrinogenemia and elevations of serum gamma glutamyl transferase that were thought to reflect liver cell growth; neither was AFP elevated in these patients during intervals of suspected liver cell degeneration as judged by elevated levels of serum enzymes. Elevation of serum AFP may require a greater degree of liver cell regeneration than is usually seen in chronic liver diseases such as cirrhosis.

Serum AFP levels were measured in 12 patients with massive hepatic necrosis. AFP became elevated only after 10 days or more following the liver destruction, but did so in a survivor as well as in those who died. Patients dying less than 10 days after liver injury did not have elevated AFP levels.

2. Metabolism of AFP (and HCG): Following precedures developed for studying in vitro synthesis of AFP in monkey hepatomas, experiments are now in progress to study the synthesis of both AFP and HCG by germ cell testicular tumors. The initial short-term in vitro cultures of this tumor tissue indicated the synthesis and release of AFP, HCG or both. The synthesis rates could be used to determine an estimated number of tumor cells which could then be verified by the quantity of tumor removed by "de-bulking" surgery. Such information should allow the amount of residual tumor to be determined by post-operative marker levels and thereby provide valuble guidance for the necessary adjuvant chemotherapy.

3. AFP in Combination with Other Markers: Determinations of CEA and HCG in addition to AFP were made using the frozen serum samples antedating the diagnosis of cancer for 9 pancreatic and 8 gastric carcinoma patients from the Framingham Heart Study. Control subjects were matched by age, sex, smoking status and pulmonary vital capacity. Despite 20% "false" positive CEA and HCG elevations, which were found in smokers with low vital capacities and post-menopausal women respectively, there was evidence that the markers could be helpful in establishing an earlier diagnosis when used with other diagnostic tests. Double marker elevation (CEA and HCG or CEA and AFP) was found only in the cancer patients indicating the increased specificity obtained by two or more simultaneously elevated markers. Another collaborative study will examine and compare the same three markers in the prediagnosis sera of patients with gastric and pancreatic carcinoma, along with sera from control patients with acute and chronic, non-malignant gastric and pancreatic disease.

4. Study of AFP in Primates: Continuing carcinogenesis studies in 1-nitroso diethylamine (DENA) treated monkeys have used serum AFP to monitor induction of hepatocellular carcinomas. Monkeys receiving doses of 40 mg/kg IP developed tumors in less than 1 year, animals receiving 10-20 mg/kg had a latent period of 1-1.5 years while those receiving 5 mg/kg have taken

4-5 years to develop tumors. Monkeys treated with DENA at 1 mg/kg have not developed tumors after more than 5 years of treatment. There is some indication that monkeys receiving higher doses of DENA have higher levels of AFP associated with the tumor.

A small proportion (approximately 10%) of DENA treated monkeys with hepatocellular carcinoma and abnormal serum AFP levels, have shown a significant decline in serum AFP concentration in the absence of therapy or any other known intervention. This has been observed in 3 different species of monkeys and in animals receiving DENA at doses of 10 or 40 mg/kg every 4 weeks. The decline in concentration has occurred more than once in some animals and represented a decrease of as much as 99.9% of the serum AFP. However, none have fallen into the normal range, and none have remained at the lower level for longer than 2 months. We have speculated that such episodes may reflect spontaneous tumor regression followed by re-growth. We plan to prospectively study such animals during the episodes of AFP decrease to determine if immune mechanisms might be involved. The study will include: 1) biopsy and histologic examination of liver nodules, 2) testing for circulating antibody to AFP or immune complexes and 3) cellular cytotoxicity and lymphocyte stimulation assays using peripheral blood leukocytes and autologous and heterologous tumor cells.

Monkey (Mk) AFP was purified from both fetal and hepatoma souces and demonstrated no detectable immunological or physicochemical differences. Similarly isolated human AFP had similar physicochemical characteristics to the Mk AFP, but when compared in the RIA for human AFP showed marked antigenic dissimilarity.

Antisera to Mk AFP was produced in a rabbit (R-211) and a double-antibody RIA for Mk AFP was developed with the antiserum diluted 1:30,000 giving around 35% maximum binding. The sensitivity of this RIA was 6-10 ng/ml. Previously we had used a modified RIA for human AFP (standard curve developed with Mk AFP) which had certain inherent inaccuracies at the extremes of the standard curve and also lacked sensitivity. In the process of surveying a large number of different species of primates with the new Mk AFP assay, an unsuspected hepatoma was diagnosed in a monkey, removed by surgical treatment and the monkey has been monitored post-operatively by serum AFP.

5. Study of AFP in Mice: The C3H-A^{vy}fB mouse strain has a high incidence of spontaneous hepatocellular carcinoma. We have utilized this strain as a model for monitoring the development of hepatoma by the serial measurement of serum AFP. In order to perform this study it has been necessary to develop the reagents and techniques of a sensitive RIA for murine AFP. The sera are now being quantitated and results will be correlated with the histologic findings of autopsies done at various times during tumor development.

Three transplantable mouse hepatoma lines have been established for studies on tumor load estimated by serum AFP value, immunotherapeutic trials and chemotherapy screening for active agents.

B. Studies of Lung Tumor Associated Antigens (LTAA) Objective 5, Approach 4

1. Isolation of LTAA: A human LTAA was purified from the saline extract of an adenocarcinoma of the lung, using standard biochemical procedures after the addition of a trace quantity of radio-labelled LTAA from a different tumor to facilitate recovery of the antigen. The purified LTAA-2 was glycoprotein and contained sialic acid; the molecular weight was 76,000, composed of 3 sub-units of 25,000. Physical properties were: Stoke's radius, 39.4 Å; $S_{20,w}$, 4.24S; $D_{20,w}$, 5.15×10^{-7} $cm^2 sec^{-1}$; and a frictional ratio of 1.40. The purified and radioiodinated LTAA-2 had apparently identical properties with the unlabelled antigen and retained immune reactivity. All of these findings support the conclusion that an identical LTAA has been isolated from two different human lung cancers.

2. Development of a RIA for LTAA-2: Following isolation of adequate amounts of LTAA-2, the methodology for ^{125}I-labelling was developed and double-antibody binding titration of the absorbed antiserum (R-201) showed that a final dilution of 1:9,000 would precipitate 30-40% of the labelled antigen. The technique of RIA for the LTAA2 was the same as that utilized for AFP. In preliminary results comparing normal sera with sera from patients with active lung cancer (not yet analyzed by histology or stage) there appeared to be a considerable difference in the quantitative levels of these two groups. Unfortunately, there is material in normal serum that competes for binding with the LTAA-2 although the material is probably present in all human sera, cancerous as well as normal. Further refinement of the RIA will require identification of the serum competitor and exclusion or removal of this effect.

3. Isolation of competitor in normal human plasma: Utilizing the RIA for LTAA-2, the region (fractions) of normal plasma with competing activity have been separated by sieving chromatography on Sephadex G-200 and then by preparative electrophoresis on polyacrylamide gel. The components of these fractions are being identified by antisera specific for a large number of serum proteins and the competitor will be identified by subtraction from plasma by the specific antisera. The isolated competitor will be compared with LTAA-2 and will be used to further absorb the "specific" antiserum used in the RIA.

4. Immunolocalization of tumors: To identify and isolate antibodies to human cancer cell surface antigens, a radiobinding assay was developed. Target cells were fixed in 3% glutaraldehyde and antibody binding was quantitated with radioiodinated staphyloccal protein A. With this method several antisera to human lung cancer antigens have been screened and one of these has been absorbed in multiple steps to remove binding activity with normal cell types. This technique is sensitive, convenient and quantitative and allows a method for the careful absorption of antisera to remove unwanted cross-reactivity. The same technique can also be used as an affinity method for purification of antibody with selected specificity. Such isolated antibodies can be radiolabelled and potentially could be used for localization and detection of sub-clinical tumors.

5. Immuno-staining of human lung cancer: The technique of immunoperoxidase cellular staining can be performed on fixed and embedded tissue and has been shown to correlate well with elevations of serum markers in germ cell testicular tumors. A preliminary survey of 36 lung cancers with 5 different antisera have shown that all were stained with anti-CEA and 32 with anti-LTAA-2. Only 6 of 18 squamous cell carcinomas and 2 of 4 large cell carcinomas were stained with anti-ACTH, none of the 6 adenocarcinomas or 8 small cell carcinomas were positive with this reagent. Anti-HCG stained only one squamous cell carcinoma and the anti-AFP did not stain any of the lung cancers. Further work on the technique should improve both sensitivity and specificity.

C. Studies of Other Human Tumor Associated Antigens

1. Breast cancer associated antigen(s): A human breast cancer tissue culture line (MCF-7) has been grown in large amounts to use for isolation of surface membrane antigens to be detected by the leukocyte migration inhibition (LMI) assay and also by raising antisera to the antigen(s). Plasma membranes have been isolated by partition between two immiscible polymer phases, monitored by phase contrast microscopy and 5'-nucleotidase activity. We are currently analyzing proteins removed from the membranes by lithium diiodosalicylate and Triton X-100 for LMI activity and also for antigenicity in immunizing rabbits for antiserum.

II. Tumor-Associated Surface Antigens (TASA) in Mice. Objective 3, Approach 5

A. Demonstration of Surface Antigens from a Moloney Leukemia Virus Induced Tumor (MBL-2)

1. Immunochemical characterization of TASA from MBL-2: The surface proteins of MBL-2 cells were radio-iodinated and then extracted by Nonidet P-40. The solubilized materials were then reacted with a variety of antisera to murine leukemia viral proteins (gp 69/71, p30, p15, p12, and p10) produced in rabbits and goats with sera from (MSV) C57BL/6 mice which rejected a murine sarcoma virus (MSV)-induced tumor, and with rabbit anti-MBL-2 serum. The resulting radio-precipitates were analyzed and compared by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Anti-gp69/71 was the only anti-viral protein sera which was active, detecting a "doublet" peak of 70,000 and probable degradation fragments of 42,000 and 35,000 daltons. Anti-MSV showed a pattern very similar to that seen with anti-gp69/71, indicating probable reactivity with viral envelope antigen, gp69/71. Anti-MBL-2 reacted with a cell surface antigen of 95,000 daltons which did not appear to be related to virion antigens. Reactivity of this antisera was not affected by absorption with disrupted M-MuLV or with gp69/71 and p30 purified from M-MuLV. The 95,000 dalton antigen appeared to be glycoprotein in nature since it was also demonstrated when cells were labelled with ^{3}H-glucosamine.

2. Isolation of MBL-2 TASA: Antigens were recovered by detergent extraction (NP-40, sodium deoxycholate and octyl β-glycoside) and by a polyethylene glycol-dextran (twophase) method for the isolation of cell membranes. Detergent extracted antigens were demonstrated by inhibition of complement dependent antibody cytotoxicity against MBL-2 target cells. The membrane preparation and the NP-40 extract were active when tested for antigen by the migration inhibition assay. Further purification of TASA is now in progress using various immunochemical procedures.

III. Development of quantitative assays for factors influencing cell-mediated immunity

A. Quantitative Assays for Lymphokines

1. Immunochemical studies on murine macrophage migration inhibition factor (MIF): Fucose affinity columns have been used for the preliminary enrichment of MIF activity, but actual protein yields have been very small. Radioiodination of this enriched fraction resulted in very heterogenous labelling and only a small per cent of the material showed binding to a macrophage cell line (WEHI-3). Current efforts are directed at obtaining a larger amount of protein with MIF activity so that subsequent physical isolation steps may be performed.

2. Immunochemical studies on human leukocyte migration inhibition factor (LIF): Fucose affinity columns have also been used for enrichment of LIF activity from the supernatant fluid from cultured lymphocytes induced to produce LIF. By sieving chromatography on Sephadex G200 the LIF activity was slightly smaller than serum albumin, which agrees with other reports. Studies are in progress to define the carbohydrate binding characteristics of LIF so that more specific and/or efficient methods of isolation might be utilized.

Significance for Biomedical Research:

There are now many circulating substances identified which can be assayed in the blood of individuals with neoplastic diseases which are reported to be associated with the tumor process. Some of these substances are much more specific for malignancy than others. Those substances that are the most specific have the potential for being utilized as aids to early diagnosis (possibly for screening) and for localization of tumor, but also have the immediate advantage in serving as tumor markers to monitor the effectiveness of therapy. Most visceral, solid tumors have a common disadvantage in not being easily measured or otherwise evaluated for progression or regression. This laboratory is committed to the clinical application of known tumor markers (AFP, HCG and CEA) as well as to the discovery of new ones (LTAA). In addition to demonstrating an association between certain tumors and markers, we are interested in studying the metabolism of the markers to further define the precise relationship. It is possible that the tumor markers or antisera directed against them could have some value in immunotherapy or even in the preventive aspects of cancer. This research on tumor markers falls under

Objective VI, Approaches 1 and 2 and Objective V, Approaches 2, 3 and 4 of the National Cancer Plan.

The study of cell surface antigens and ways to detect and quantitate them will provide basic information needed to understand the process of carcinogenesis and malignant transformation. Animal models and in vitro systems are used because of the ease of manipulation and control of variables. We are searching for membrane antigens since those on the surface of the cell are the most likely to trigger immune recognition, serving as targets for immunologically directed therapy or radio-antibody localization of small foci of metastatic tumor cells. This research falls under Objective III, Approaches 4 and 5.

Proposed Course of Project:

This project represents the continued commitment of our laboratory to immunodiagnosis utilizing circulating antigens (released or secreted by tumor cells) and cell surface antigens. We will continue to derive information as to their usefulness in following the course of malignant disease and how they may be used in combination to aid the early diagnosis of cancer.

We will continue to use AFP, HCG and CEA measured by sensitive RIA to monitor patients with hepatocellular pancreatic, gastric, colonic and testicular carcinoma. We will use the carcinogen-induced hepatomas in primates to study the relationship of AFP to tumor burden by measuring synthetic rates and catabolic rates of AFP in the animals with tumors. We will continue similar metabolic studies in patients with testicular germ cell tumors utilizing HCG in addition to AFP. We will continue to perform tissue staining for a wide-variety of tumor associated antigens as an aid to classification, staging, prognosis, therapeutic choice and tumor biology of several different cancers.

We will be working on isolation of antibodies to cell surface antigens which can then be radio-labelled to facilitate the localization of small tumor nodules by computerized axial tomography or radio-scanning.

We will continue to work on identification and isolation of tumor-associated antigens in mouse tumors to extend our basic knowledge of the process of transformation and of the host response to tumor. In addition we will be comparing the humoral and cellular immune responses to the same isolated antigens.

Efforts will continue to isolate the antigen(s) in human breast cancer which may be responsible for the demonstrated cellular immune response in some patients with this tumor.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08517-03 LID

PERIOD COVERED

October 1, 1978 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cellular and Tumor Immunity in Experimental Animal Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Laboratory of Immunodiagnosis

SECTION

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7	6	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Cellular and tumor immune responses were examined in animals injected with C. parvum or in tumor bearing or tumor immune mice. T lymphocytes from spleens of mice injected 14 days earlier with murine sarcoma virus (MSV) produce migration inhibition factor (MIF) when stimulated with intact tumor cells but T cells isolated from the tumor do not. Macrophages isolated from the tumor are capable of suppressing stimulation of MIF production by MSV immune spleen cells. Cytolytic macrophages can also be isolated from the tumor and represent a heterogeneous population of cells as noted by 1 g velocity sedimentation studies. Differences in subpopulations and levels of activity were observed in macrophages isolated from regressing and progressing tumors and from tumors in nude mice. Addition of endotoxin to the assay increased the levels of macrophage mediated cytotoxicity. Macrophage mediated cytostasis of tumor cells and lymphocytes could be demonstrated in both nude and conventional mice injected with either MSV or C. parvum. Suppression of lymphoproliferative responses by macrophages from tumor-bearing mice could be partially reversed when prostaglandin synthesis was inhibited. Macrophage tumor lines have been shown to exhibit cytostasis and cytolysis. Attempts have been made to establish long term T cell lines with cytolytic activity.

PHS-6040
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Project Description:

Objectives: The objectives of this project are: (1) to detect tumor-specific and tumor-associated antigens on malignant cells; (2) to detect cellular immune reactions to tumor antigens; (3) to dissect the multifaceted immune response to tumor growth; (4) to develop and standardize *in vitro* assays to measure cell-mediated immunity to tumor antigens; (5) to determine the interrelationship between the different subpopulations of leukocytes in the generation of anti-tumor immune responses; (6) to study functional activities of lymphoid cells within the tumor, especially with regard to their anti-tumor activity; (7) to correlate the findings of the *in vitro* assays with the growth of the tumor in the host; (8) to evaluate the diagnostic, prognostic and therapeutic significance of cellular immune responses to tumor antigens and to evaluate the effect of tumor substances (such as antigen, antibody and antigen-antibody complexes) on these cellular immune responses; and (9) to measure general immunological competence of tumor bearing animals, and to determine the relationship between this and the growth of the tumor.

Methods Employed: Host lymphoid cells were obtained from the spleen, lymph node, peritoneal cavity, peripheral blood, and thymus (and tumor of tumor bearing animals) of immune and control animals. A variety of normal and tumor cell lines were maintained in suspension culture. Normal T lymphocytes from immune mice were placed in long term culture employing conditioned medium. A variety of tumors induced by viral, chemical or unknown agents were maintained by transplantation in syngeneic animals. Immune and normal lymphocytes and tumor cells were stored in a viable frozen state. Inbred strains of mice were employed for several different allogeneic and tumor model systems. Immunological competence and/or cellular immune reactivity were measured *in vitro* by the following tests: lymphocyte stimulation by mitogens, lymphocyte-mediated cytotoxicity (^{51}Cr and ^{125}IUdR release), macrophage-mediated cytotoxicity, production of migration inhibition factor (MIF), generation of primary and secondary cytotoxic responses, suppression of mitogen-induced proliferation, generation of secondary cytotoxic responses and MIF production, and inhibition of growth of lymphoma cells. Antibody levels were measured by the ^{51}Cr release assay and adsorptions were performed to render the antiserum specific. *In vivo*, immunity was assessed by adoptive transfer and by challenge experiments. Animal tumor cell membranes and soluble fractions were prepared for antigenic study. Lymphoid populations were enriched or depleted of different subpopulations by iron/magnet treatment, passage over adherence columns, treatment with antisera plus complement, or adsorption on antigen or antigen-antibody monolayers. Further cell separations were performed based on cell size using 1 g velocity sedimentation.

Major *Findings:*:

I. Cell-Mediated Immunity in the MSV Tumor System

Background

Murine sarcoma virus (MSV) when injected into normal mice produces tumors which appear 7-9 days after injection, reach peak size at 12-13 days, and regress by 21-25 days. Several different T cell-mediated immune responses can be measured in these animals. Primary cytotoxic reactivity (as detected by 4- or 18-hr ^{51}Cr release cytotoxicity assays) peaks 14 days after injection. In addition, production of migration inhibitory factor (MIF) and proliferation of spleen cells in response to stimulation with cell-associated tumor antigen also peaks 14 days after inoculation of virus. On the other hand, secondary cytotoxic responses can be observed in vivo after challenge and in vitro after stimulation for periods over 100 days after virus injection. Two different macrophage functions have also been reported previously in this system. Tumor-bearing mice have activated macrophages that are capable of inhibiting tumor cell proliferation (cytostasis) and of suppressing the proliverative response of normal spleen cells to PHA, Con A and LPS. In addition, macrophages from tumor bearing mice exhibit nonspecific cytolytic activity against tumor cells.

A. MIF Production

Previously we have shown that spleen and lymph node cells from mice 9-17 days after injection with MSV can be stimulated to produce MIF with intact tumor cells or with soluble antigen. The latter requires the presence of macrophages compatible at the IA subregion of the major histocompatibility complex while the former doesn't depend on macrophages. In an effort to determine whether this same activity could be detected in lymphocytes from the tumor, host cells were isolated from MSV tumors obtained 14 days after inoculation by enzymatic disaggregation with collagenase and DNAse, and stimulated with intact tumor cells. MIF production was not detected either in the whole cell population or in the cells depleted of macrophages by rayon wool fractionation or the carbonyl iron/magnet technique. These preliminary observations led us to consider the possibility that a suppressor phenomenon might be responsible or at least involved in the unresponsiveness of the lymphocytes from the tumor. To test this hypothesis we set up a system in which cells from the tumor (CfT) were added as a third party to a MIF producing system. Spleen cells from mice 14 days after injection of MSV were depleted of macrophages (d-ISC) and stimulated by RBL-5 tumor cells. MIF production was suppressed when 2-6% of the CfT were added at the beginning of culture. Using plastic adherent cells from the tumor (aCfT), suppression was seen using even lower concentrations of cells. If, on the other hand, phagocytic or adherent cells were removed before the CfT were added, no suppressor activity was observed suggesting that the suppressor cells were macrophages. T cells were not required for suppression since pretreatment of the CfT with anti-Thy 1.2 plus complement had no effect on their ability to suppress.

Since macrophages are the responding cells in the MIF assay and therefore may have receptors for MIF, it was possible that the suppressive effect was due to the adsorption of the synthesized lymphokine on the activated macrophages. However, incubation of MIF containing supernatants with the CfT did not affect the MIF activity. Moreover, when standard MIF rich supernatants were titrated by diluting then in normal medium or in supernatants derived either from suppressed cultures or from a-CfT cultured alone, no differences in the titer of MIF activity was observed. These results indicate that the suppressor activity did not reflect an inactivation or degradation of MIF activity by a soluble material nor was MIF adsorbed to the suppressor macrophages. It is interesting to note with regard to the latter statement that macrophages from the tumor did not respond to MIF even though they migrate very well.

It does not appear that the macrophages turn off a cell once it is producing MIF but rather that they interfere with the induction of MIF production. Macrophages were able to inhibit MIF synthesis only when they were added to the MIF producing system during the first 6-8 hours of culture. MIF production was not affected if they were added at later time points. Interestingly, it was at about 6-8 hours after the initiation of the culture when MIF activity was first detected. Preliminary experiments showed that suppression of MIF production was an irreversible phenomenon and could occur in the absence of antigen since after removal of macrophages from suppressed cultures, irrespective of whether antigen had been previously added, MIF production could not be induced.

Suppression of MIF production was not antigen specific. Adherent cells from MSV tumors were capable of inhibiting MIF production not only by MSV immune spleen cells but also by alloimmune spleen cells and by normal spleen cells stimulated by the mitogens Concanavalin A or PHA. Furthermore, macrophages from normal mice injected with BCG or Corynebacterium parvum were also capable of suppressing MIF production by MSV immune spleen cells. Hence, macrophages activated by various stimuli were capable of nonspecifically inhibiting the stimulation of MIF production by both immune and nonimmune spleen cells.

Suppression of immune functions by macrophages has previously been associated with the ability of the macrophages to inhibit proliferation of lymphocytes. We confirmed that MIF production was not dependent on the proliferation of the responding T cells and that the suppression could also be observed when proliferation of the T cells was inhibited. Therefore, macrophages appear to be able to suppress proliferation independent functions as well as the proliferation of lymphocytes.

B. Macrophage-Mediated Cytolytic

We have continued our studies on macrophage-mediated cytotoxicity induced by the injection of MSV. Macrophages recovered from the spleen, peritoneum or from the tumor of mice injected with the regressor stock of MSV were nonspecifically cytolytic as measured by an 18 hour ^{51}Cr release cytotoxicity assay. Studies conducted during the past year have concentrated on the macrophage-mediated cytotoxicity of cells isolated from tumors induced either in conventional or nude mice by the injection of either regressor or progressor MSV virus.

Cytolytic macrophages recovered from the tumors of mice injected 14 days earlier with either regressor or progressor stock of MSV had comparable levels of cytotoxicity. However, macrophages from progressively growing tumors, at 50-60 days after inoculation of the virus, had lower levels of activity. Nude mice on the other hand did not have cytolytic macrophages in their tumors. The differences in the level of macrophage mediated-cytotoxicity could not be accounted for by the differences in the percentage of macrophages in the tumors since the testing was done with adherent cells from the tumor. Nevertheless, the regressing tumors contained about 50% macrophages as opposed to about 45% in the progressing tumors in the conventional mice and about 35% in the progressively growing tumors in the nude mice.

Other differences were noted in the macrophages derived from the different animals when 1 g velocity sedimentation studies were performed on the cells isolated from the tumors. When macrophages were identified by latex particle ingestion, regressing tumors had two or three different subpopulations, one sedimentating at about 4 mm/hr, and another at 6 mm/hr and sometimes a third at 7-8 mm/hr. The profile from progressively growing tumors from nude mice were deficient in the macrophages sedimenting at 4 mm/hr while they still had larger macrophages (a population that sedimented at 6 mm/hr). Effector cell function distributed a little differently. In the tumors from regressor mice, there were two peaks of reactivity corresponding to the main peaks of macrophages as identified by latex particle ingestion. However, there was functional activity only in the cells sedimen ting at 4 mm/hr. Nude mice, even after separation by velocity sedimentation, exhibited no macrophage mediated cytolysis.

C. Enhancement of Macrophage-Mediated Cytotolysis

Endotoxin (LPS) when added directly to the cytotoxicity assay has been shown by other laboratories to enhance macrophage-mediated cytotoxicity. We have examined the ability of this substance, along with other materials, to increase the level of activity of macrophages isolated from regressing and progressing tumors in conventional mice and from progressing tumors in nude mice.

We found that LPS could enhance macrophage-mediated cytotoxicity when added directly to the assay, even at very low concentration (1 ng/ml). Adherent cells from the tumors of mice 14 days after injection with either the regressor or progressor viruses had similar levels of activity without the addition of LPS and showed similar increases in activity when the assay was performed in the presence of this material. On the other hand, the cytotoxic reactivity of adherent cells from mice with progressively growing tumors that had lower levels of cytotoxicity initially, could be augmented by the addition of LPS but the level of activity did not reach that attained with the cells harvested at 14 days, even when higher levels of LPS were employed. Macrophages from tumors induced in nude mice could not be stimulated to be cytolytic by the addition of LPS.

To determine whether the enhanced reactivity that was observed after the addition of endotoxin was the result of augmentation of the cytotoxic potential of the cells that were already activated or was due to induction of cells that were initially unreactive, 1 g velocity sedimentation studies were performed. Cells from tumors induced by regressing or progressing virus in conventional mice or from progressively growing tumors in nude mice were fractionated and each fraction was tested for reactivity with and without the addition of LPS. The distribution profile of the cytotoxic reacitivity was identical in all cases regardless of whether the LPS had been added to the assay. Hence it would appear that addition of LPS to the assay was only augmenting the reactivity of the cells that were previously exhibiting cytotoxic reactivity and not activating cells that had no reactivity.

Several other agents were tested for their ability to enhance macrophage-mediated cytolysis. We found that poly I:C and muramyl dipeptide, a synthetic preparation of a constituent of BCG, could enhance reactivity as well as LPS, while dextran sulfate and zymosan had weaker stimulatory effects. Furthermore, addition to the assay of a crude preparation of interferon, derived from fibroblasts causes similar dose-responsive stimulation of cytolytic activity.

D. In Vitro Secondary Cytotoxic Response

Spleen cells from MSV immune mice could be stimulated in vitro to undergo a secondary cytotoxic response. Low levels of proliferation were associated with this response and by inhibition proliferation with a hot thymidine pulse the generation of reactivity was almost completely blocked. The role of macrophages was examined in the generation of this response, both as helper cells and also as suppressor cells.

High levels of cytotoxic reactivity could be generated when MSV immune spleen cells were stimulated with tumor cells in vitro. However, if macrophages were extensively depleted before the cells were stimulated no cytotoxicity was detected at the end of the culture period. The response could be restored by adding back syngeneic macrophages to the depleted spleen cells before they were stimulated but not by the addition of allogeneic macrophages or 2-mercaptoethanol. Similar macrophage requirements for the generation of a primary allogeneic cytotoxic response could also be shown. However, in this system, allogeneic macrophages could serve as the source of antigenic stimulus in the absence of syngeneic macrophages.

For the syngeneic response to tumor antigens, viable macrophages were required. However, macrophages that had been treated with mitomycin C to block replication were able to function as helper cells in this assay. Mapping studies indicated that similarity at the major histocompatibility complex was essential for the macrophages to act as helper cells and, as previously shown for the macrophages needed to help in the stimulation of MIF production in response to soluble tumor antigen, compatibility at the IA subregion appeared to be required.

Spleen cells from mice that had progressively growing tumors responded very poorly when stimulated in vitro. However, if the macrophages were depleted and normal, syngeneic macrophages were added back, significant levels of reactivity were generated. If spleen cells from mice with a progressively growing tumor were added to a secondary response culture of spleen cells from regressor mice, the generation of the cytotoxic response could be inhibited, indicating the role of suppressor macrophages in this system.

E. Attempts to Establish Continuous T Cell Lines

In the past we have generated cytotoxic T lymphocytes in vitro after a secondary stimulation of spleen cells obtained from an immune animal. If left in culture these cells would eventually die and the length of time during which these cytotoxic lymphocytes could be studied was limited. We have attempted to extend the culture life of these cells, through the use of conditioned medium (CM) as has been reported by other laboratories, to eventually establish continuous T cell cultures (CTC). Conditioned medium was prepared by stimulating normal mouse or rat spleen cells with Concanavalin A and harvesting the supernatant 24-28 hours later. The cells to be established in culture were MSV-immume spleen cells that were stimulated in vitro to undergo a secondary response. Several important findings have been made in our attempts to establish CTC with specific cytotoxic activity. Production of the conditoned medium is critical in establishinging and maintaining the CTC. An assay has been set up to analyze the CM to determine the relative activity of different batches. Basically, CTC were suspended in fresh medium and seeded in microtest plates. An equal volume of different dilutions of CM was added to each well and the next day the plates were pulsed with ^{3}H-thymidine for four hours and harvested. Supernatants containing CM stimulated proliferation of the CTC. Using this assay it was shown that CM produced by mouse or rat spleen cells could be used to support the growth of mouse CTC. CM produced by human cells did not appear to cause proliferation of the CTC. Currently we are attempting to demonstrate whether CM can be generated using several different continuous cell lines.

We have been able to keep spleen cells in culture for up to 2-3 weeks. The growth characteristics of the different lines that were established for these intervals varied. Our first cell line grew very well initally, could be seeded at relatively high concentrations (10^5 - 5×10^5/ml) and would grow to concentrations of over 10^6/ml. However, our more recent attempts have shown that the cell concentration must be lower, with the cells seeded at about 10^4/ml and grown to concentration of no higher than 10^5 - 2×10^5/ml. Our secondary cultures were established on day 0 and on day 2 half of the culture medium was replaced with CM. This appeared to give better results in initiating the CTC. At day 5 the cells were harvested and seeded at 5×10^3 - 5×10^4/ml in 50% CM. The cultures were fed every two to three days. In agreement with other laboratories, our CTC exhibited cytotoxic activity. However, preliminary evidence suggests that some of the cytotoxicity was not specific for the antigens used to simulate the culture.

Currently we are attempting to establish continuous CTC that will grow for longer periods of time. In addition experiments are planned to further characterize the CTC by using surface markers and to determine the cytotoxic reactivity of these cultures.

II. Marcrophage-Mediated Suppression and Cytotostasis in the MSV and Other Systems.

A. Macrophage Suppression in Nude Mice

Studies have been continued on the relative role of T cells in macrophage-mediated inhibition of tumor cells growth *in vitro* and in suppression of mitogen induced lymphoproliferation. Employing the growth inhibition assay (GIA) to measure tumor cell cytostasis by macrophages, dose response studies using various amounts of *C. parvum* ip into BALB/cN nude mice showed that 2.1 mg was the optimal dose. Kinetic studies demonstrated that GIA first appeared in spleen cells from *C. parvum* mice around day 3, decreased on day 5 and peaked again at days 7-9, then in most cases declined rapidly to unreactivity by day 12. In some instances the activity decreased more slowly through day 14. However, in no circumstances was GIA demonstrated after day 15. Similar results were obtained when C57BL/6 mice were injected with *C. parvum* but the reactivity was generally higher in these animals. When MSV was used to stimulate suppressor cell, the kinetics were quite similar to the *C. parvum* curve. However, the second major peak of reactivity was found at 10-12 days rather then at 7-9; all activity had disappeared by day 14. The activity appeared to increase with tumor size but for some reason the activity dropped after day 12 even though the tumor was growing progressively. The earlier peak seen at day 4-5 could have been natural killer activity, however, this was not tested experimentally.

Previous studies have demonstrated that spleen cells from nude mice injected with *C. parvum* were capable of suppressing the LPS response in nude spleen cells. In order to answer the question as to whether these cells were capable of suppressing the mitogenic response in conventional mice and also the proliferation of tumor cells *in vitro*, experiments were done using the same source of suppressor cells in the growth inhibition assay and in the mitogenic suppressor assay where conventional spleen cells served as responders. Nude spleen cells from mice injected with *C. parvum* 9 or 12 days previously were able to suppress the LPS response in nude and conventional mice, but much less effectively in the latter. However, they were unable to suppress the response to PHA or Con A in conventional mice even though they were able to inhibit the proliferation of tumor cells in the GIA. These results would suggest that there may be some specificity in the anti-proliferative effects of the macrophages and studies are under way to examine this hypothesis.

Since the BALB/cN nude mice were all from heterozygous mothers (Nu/+), it was conceivable that their T lymphocytes were exposed to some circulating or milk-born T cell factors from their mothers and that these cells cooperated with the macrophages in generating suppressor and growth inhibitory activity. To answer this question, NIH Swiss nude mice from homozygous (Nu/Nu) mothers, heterozygous (Nu/+) mothers, the littermates (Nu/+) of the latter along with conventional NIH Swiss were injected with C. parvum and 9 days later the spleen cells were assayed in the GIA. In all cases, the conventional mice and the heterozygous littermates of the nude mice from heterozygous mothers responded equally and gave high levels of GIA (>90%). Swiss nudes from homozygous and heterozygous mothers developed fairly strong inhibitory activity (70%), with homozygous nudes in some experiments being slightly lower than the nudes from heterozygous mothers. The GIA was mediated by an adherent cell, probably a macrophage. Esterase staining of the spleen cells from the C. parvum injected mice indicated that 40-50% of the cells were positive and even after deplation of adherent cells, 25% of the cells were still positive. Additional characterization of these cell populations are currently being performed.

Whether C. parvum or MSV was used as the stimulator of suppressor cells, two phenomena were consistently observed in the above studies. One was that the ability of nude spleen cells to suppress either proliferation of tumor cells or LPS induced blastogenesis was always greater than that of the corresponding conventional mice. The second was that the overall level of suppression was very low, around 30-40% in C. parvum nude spleen cells and <20% in conventional mice spleen cells. Frequently it was difficult to exhibit any suppressor activity at all in BALB/cN conventional mice. Studies were initiated in an attempt to determine the cause for the excessively low response in both mice, especially in the conventional mice, as these findings were quite unusual and unexpected.

B. Strain Differences in Response to C. Parvum

Several possibilities were entertained as to why such low suppressor and growth inhibitory responses were seen in BALB/cN mice. Experiments were performed to verify that the A/T ratios being used were optimal for working with nude spleen cells. To determine whether the current preparation of C. parvum was satisfactory, experiments were performed using C57BL/6 mice, since previous work performed in this laboratory with C. parvum had been with this strain. High levels of suppression (80-90%) were observed in these studies. This suggested that the BALB/cN mice were deficient in manifesting this activity and studies were performed to see if this was genetically determined. Initial experiments have suggested that BALB/cJ mice responded slightly lower than several other conventional strains (C57BL/6, CBA/J, A/JAX and AKR) but not as low as previously observed with the BALB/cN. Studies are currently in progress to repeat this experiment with an even wider variety of strains of mice. Recent experiments utilizing conventional BALB/cN mice from three (3) different animal farms have supported the hypothisis that the BALB/cN are indeed low responders and that the low response is not due to environmental factors.

C. Effect of Indomethacin on Macrophage Suppressor Cell Activity

Studies have recently been undertaken to explore the possible mechanisms by which macrophages may mediate their suppressor activity. Indomethacin, an inhbiitor of prostaglandin synthesis has been used in experiments to determine whether macrophages are mediating suppression through the secretion of prostaglandins which may be inhibitory. There have been recent reports to this effect for human monocyte suppressors. Indomethacin was present in the assays during their entire time of incubation. In studies using 1μg/ml indomethacin in assays of suppression of lymphocyte proliferation to mitogens in mice, the indomethacin was only effective when low levels of suppression were present. That is, when suppression was present at levels of 70-80%, indomethacin had no effect. However, when the level of suppression was 30-40% or below, indomethacin would block 50-100% of the suppression. Attempts to use higher concentrations of indomethacin have not demonstrated any greater effectiveness. Studies looking at the effect of indomethacin on the GIA in mice have not shown any positive results. This may be due to the nature of the assay and the radionuclide used in that the level of inhibition as a function of A/T ratio showed a very precipitous drop as the A/T ratio decreased. Thus at one A/T, we have obtained 80-90% inhibition and no effect of indomethacin, and at a 2 fold lower A/T level of inhibition might be 0. Therefore we have not been able to obtain a moderate level of inhibition which may be susceptible to indomethacin, as was seen in the mitogenic stimulation suppressor assay. Various macrophage tissue culture lines available in our laboratory (PU5-1.8, WEHI-3, P388D_1 and J744) have all been shown to produce prostaglandins. However, preliminary experiments using indomethacin have not demonstrated that indomethacin has any effect on their inhibitory activity.

Further studies are presently in progress to investigate ways to enhance the effectiveness of indomethacin and to look at its role in vivo in the MSV system. Initial studies into its effects on other cell-mediated reactions as MIF and macrophage mediated cytotoxicity have not been definitive and are being further investigated.

D. Characterization of Macrophage Subpopulations

Fractionation of populations of peritonal exudate cells (PEC) by 1 G velocity sedimentation has been done as part of an overall study to compare the subpopulations fuctionally, particularly in regard to suppressor cell and macrophage-ADCC activity. Studies have shown that when PEC were harvested on days 3,4, or 5 after thioglycollate the sedimentation profile was basically the same with two peaks, one at 4.0 ± 0.5 and the second at 5.5 ± 0.5 mm/hrs. The percent activity per fraction was not consistently greater in either peak; however, in the majority of experiments it was greater in the small, faster sedimenting cells. When esterase stains were done on all fractions, the percent esterase positive cells was found to be maximum in the fractions corresponding to the first peak of GIA activity. When latex ingestion was evaluated it was found that fractions with maximum esterase and GIA activity are low in latex ingesting cells and that the maximum latex ingestion was in cells sedimenting at a velocity greater than 5.0 mm/hr. Very large cells (SV$\geq$7.0) showed high esterase and latex ingestion activity. Thus it would appear that the macrophages are heterogeneous in size, in esterase staining qualitites and in phagocytic abilities. Studies are under way to characterize these cells further with regard to their functional activities.

III. Studies on the Surface Characteristics of Natural Killer Cells

Natural killer (NK) cells are a subpopulation of lymphocytes found in normal mice that are capable of lysing a wide variety of tumor cells and some normal cells. These cells may play a role in immune surveillance against tumors and in the immune response against other types of viral and microbiolagical agents. Previously we have examined some of the surface characteristics of these cells and found that they have low avidity Fc receptors and low concentrations of Thy 1.2 antigen. Inasmuch as the latter surface marker is characteristic of T lymphocytes, we are currently examining several other types of receptors and/or antigens that have been found on mature or immature T cells to determine whether these can also be identified on NK cells.

In addition to the Thy 1.2 antigen, mature and immature T cells have an antigen that is also found on mouse brain. Rabbit antiserum to mouse brain, after proper adsorption, is specific for mouse T cells but the organ distribution of the cells in the mouse that are positive for this antigen is a little different than those that carry Thy 1.2. Nude mice, that lack mature T cells, have lymphocytes that are weakly positive when stained with FITC conjugated rabbit anti-mouse brain. We are trying to identify whether these same cells are the NK cells in the nude.

Many lectins have been isolated from plants and some from animals that have characteristic binding patterns to cells. Two that are especially interesting in this regard are lectins isolated from Helix pomatia (H.p.) and from peanuts both of which appear to bind predominatly to T lymphocytes. Studies are currently in progress to determine whether these lectins also bind to NK cells.

To date no uniques markers have been identified for NK cells that could be used to either specifically deplete or enrich for these cells. Identification of such markers would provide tools for identification and isolation of these cells. Antisera that react with antigens on the surface of NK cells (such as rabbit anti-mouse brain or anti-Thy 1.2) when FITC conjugated would enable separation of the NK cells from nudes by the Fluoresent Activated Cell Sorter. If specific lectins could be identified that bind to NK cells, the cells could then be isolated by rosetting techniques using erythrocytes that also bind the lectins.

IV. Macrophage Tumor Lines as Models for Functional Activity and Surface Markers of Macrophages.

Functional activity and surface characteristics of macrophages are sometimes troublesome to study because of the difficulty in obtaining populations of these cells free from contamination with other leukocytes. Recently a number of macrophage tumor line have been described that have many of the properties of normal or activated macrophages. These represent pure populations of macrophages and have proven to be very useful as models for study of macrophage activity and macrophage surface markers.

During the past year we have obtained several different macrophage tumor lines: PU5-1.8, WEHI-3, J774 and P388D1. These lines are macrophages by several different criteria and exhibit some of the functional characteristics of macrophages. However, the lines do display different properties among themselves which also make them good candidates to study the functional heterogeneity of macrophages.

All four of the macrophage tumor lines were tested for their ability to migrate out of an agarose droplet and for their ability to respond to MIF. A macrophage tumor line with these characteristics would be very useful to employ in our MIF testing and would enable us to standardize the assay. PU5-1.8, J774 and P388D1 did not migrate at all while WEHI-3 migrated as well as the normal peritoneal exudate cells that are routinely employed in the assay. Unfortunately the migration of the tumor line was not inhibitied when MIF was added but there was some evidence that there might be a subpopulation of cells that was responding. Eleven clones of the WEHI-3 were isolated in an attempt to find the subpopulation that appeared to be responding to MIF. Most of the clones (6/11) migrated well but none of them responded to MIF. In fact, there were 2 or 3 of the clones whose migration appeared to be stimulated by the supernatants that contained MIF although the cells may have been responding to other lymphokines that were also present.

The anti-tumor activity of the macrophage tumor lines was tested in two different assays, one that measures cytostasis and another that measures cytolysis. All four tumor lines could mediate cytostasis, although the levels of activity varied between the lines. When the lines were tested for their ability to lyse tumor targets in a ^{51}Cr release cytotoxicity assay, PU5-1.8 had the highest activity while P388D1 gave low levels of lysis. The other two lines did not give appreciable levels of activity against the tumor targets. We have continued to use the PU5-1.8 as a model for macrophage-mediated cytotoxicity against tumor cells and have also examined the augmentation of reactivity observed by various agents. Cytotoxic reactivity was enhanced when endotoxin, muramyl dipeptide and poly I:C were added at the beginning of the assay. Dextran sulfate and PPD would sometimes augment reactivity but the increase in activity was not as great. Preliminary experiments with a crude preparation of interferon indicated that this material can also augment macrophage-mediated cytotoxicity when added to the assay.

Another laboratory has reported that antiserum producted in a rabbit against one of the macrophage tumor lines, P388D1, after proper adsorption is capable of recognizing a specific subpopulation of macrophages (activated macrophages). Many lines of evidence suggest that subpopulations of macrophages can be recognized under a variety of conditions. We have initiated a program to produce antisera against the macrophage tumor lines to (1) obtain antibody that will recognize macrophages and (2) to prepare reagents that will recognize subpopulations of macrophages. Rabbits have been immunized with all four of the macrophage tumor lines that we are currently passaging (one tumor line per rabbit) and we are currently testing the antisera to determine their pattern of reactivity and trying to adsorb them to render them specific for subpopulations of macrophages.

Other studies that are currently in progress are designed to investigate the ability of the macrophage tumor lines to act as helper cells in stimulation of MIF production by soluble antigen and in an in vitro secondary cytotoxic response. We are also investigating the suppressor activity of these lines on lymphocyte proliferation and on MIF production. Finally, we are also employing some of the macrophage tumor lines as models to establish a macrophage-mediated antibody-dependent cell-mediated cytotoxicity assay against tumor cells.

Significance to Biomedical Research and the Program of the Institute:

The immune response to neoplasia is very complex and involves not only the interaction between host effector cells and tumor cells, but also the interaction between and among the various subpopulation of leukocytes. Some cells may serve to amplify the immune response while others may suppress it. We have been studying these interactions on several levels, both in vitro and in vivo, in order to try to determine the role of the immune response in limiting or preventing tumor growth.

Many of the studies designed to investigate the immune response to tumor growth have employed peripheral lymphoid cells. Although this is a useful method for developing in vitro tests to measure the cell-mediated responses of animals during tumor growth, the response that is taking place within the tumor is perhaps more important to the outcome of tumor growth. Hence our studies have become centered around the analysis of the functional activity of the effector cells isolated from the tumors of animals with both regressing and progressing tumors.

Our work has shown that within regressing tumors there are both T lymphocytes and macrophages with functional reactivity. The types of responses that the T lymphcotyes from the tumor exhibit are different than the responses observed with cells obtained from the spleen. Both preparation of T cells exhibit specific cytotoxicity against tumor targets but the cells from the tumor do not produce MIF when stimulated by intact tumor cells while those from the spleen do. Macrophages from both sources appear to express the same types of functional activity. Within the tumor however, there are differences in the size distribution of functionally active macrophages depending on whether they came from regressing tumors or progressing tumors. In addition, some macrophage functions are reduced or absent in cells isolated from tumors induced in nude mice. Studies on the comparison of functional activity of effector cells isolated from regressing and progressing tumors should provide valuable information on the differences in the host response and may give us insight into the reasons that the host does not deal successfully with growth of their tumors. Furthermore, if these studies could provide greater insight into the mechanism of tumor rejection, immunotherapy protocols could be established based on this information.

Macrophages are capable of modulating the host's immune response either through the ability of these to act as helper cells in the development of an immune response or by their capacity to suppress development of immune responses. In our studies we have continued to examine the role of macrophages in these two types of functions. Our investigations have shown that macrophages are required as helper cells for a secondary response in vitro. On the other hand we have found that macrophages from the tumor are capable of inhibiting the production of MIF. This is the first indication that macrophages can suppress immune functions that are not proliferation dependent and therefore has important implication on the mechanism of the interaction between macrophages and T lymphocytes. A delicate balance exists with the suppressive activity. It may be the control of this balance that ultimately determines whether the host deals successfully with invasive tumor growth. Understanding all these interactions, what influences them and how they can be modulated to the benefit of the host will give us information that we can use to inhibit the growth of a neoplasm.

Immune T lymphocytes have been shown to be important for the protection of a host against tumor challenge and for the development of immunity to tumors. However, it is not clear what T cell functions are important. We are employing several techniques to investigate this problem.. Using 1 g velocity sedimentation we have been able to identify subpopulations of MIF producing cells that respond differently after stimulation with soluble or particulate antigen. We have also identified T lymphocytes in the tumor that are cytotoxic but do not produce MIF when stimulated with particulate antigen. This gives us the flexibility to test these different subpopulations of T cells for their ability to adoptively protect mice from tumor challenge and perhaps assess the relative importance of different T cell functions in tumor immunity. Furthermore, we are attempting to grow normal, immune T lymphocytes in long term culture. This well also give us the opportunity to investigate populations of T lymphcoytes for their functional activity in vitro and in vivo.

Finally, natural killer cells may have an important role in the protection of the host from tumor induction (immune surveillance) or may assist in the anti-tumor response after different types of immunotherapy. Investigations on the nature of these cells should provide a mechanism whereby the cells can be isolated and their role in vivo assessed. Studies designed to identify the surface characteristics of these cells for this purpose are in progress.

Proposed Course of the Project:

During the next year many of the above mentioned projects will be continued and some will be expanded; work reported in previous years will also be the basis for additional studies.

Analysis of the functional activity of effector cells infiltrating the tumor will continue to have a high priority in the studies to be pursued during the coming year. Investigations into the role of T cells, NK cells and macrophages are planned. Comparisons between the cells infiltrating regressing and progressing tumors in conventional mice and the cells infiltrating the tumors in nude mice will be made. In addition, we will analyze the kinetics of the entry of the host cells into the tumor by looking at the functional activity of effector cells at different times after inocutlation. T cell function will be analyzed by looking at the ability of these cells to develop cytotoxicity in vivo and to produce MIF, MAF and interferon after stimulation with intact tumor cells and soluble antigen. Subpopulations of these populations will be examined and defined. Macrophage activity will be examined by assessing cytolytic and cytostatic anti-tumor activity and by studying suppressor activity against proliferative functions and against MIF production.

Several aspects of T cell immunity will be studied. Additional studies will be performed to investigated the role of the major histocompatibility complex (MHC) in the anti-tumor immune response. We have previously shown that the interaction between immune T cells and tumor targets in the MSV system does not require compatibility at the MHC. However, that interaction is facilitated if both share the same MHC. We plan to investigate the Ly phenotypes of the cells that are mediating the reactivity against both the syngeneic and allogeneic targets. A similar lack of H-2 restriction was found when the immune cells were stimulated to produce MIF. In addition there are different subpopulations of cells involved in MIF production depending on the form of the antigen (soluble or particulate) that is used for stimulation. Further studies and characterization on the subpopulations of cells involved in these responses are planned. Work will continue on the long term growth of normal and immune T lymphocytes in culture using conditioned medium. When long term cultures are established they will be characterized with regard to their surface markers and their functional activity both in vitro and in vivo.

We will continue to examine macrophage functions as they relate to the generation and suppression of cell-mediated anti-tumor responses. Studies will be conducted to determine the role of prostaglandins in the suppressor and anti-tumor activity of the macrophages. Suppression of MIF production will be examined to determine the possible mechanism of suppression (since it is not dependent on the inhibition of cell proliferation). In addition, the suppression of MAF and interferon production will be studied. The role of macrophages in the production and action of interferon will be examined. Work will continue on the role of the macrophage in the generation of cytotoxicity.

Natural killer activity will be studied to learn more about the surface markers present on these cells. Using this information we will then try to separate these cells to study their functional activity more closely. In addition, the role of immunoglobulin in the recognition of target cells by NK cells will be examined in mice whose immunoglobulin production has been suppressed by treatment with heterologous anti-mouse immunoglobulin.

Studies will continue using macrophage tumor lines as models of functional activity and as immunogens to look at macrophage surface markers. Using these lines we will examine the mechanism of activation of macrophages to become cytotoxic and we will compare the activity of these lines in assays that measure helper functions, suppressor activity, cytostatic and cytolytic anti-tumor functions. We will continue to look for lines that can be used in assays to measure lymphokine production.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08522-02 LID

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Development and Use of Sensitive and Specific Micro-Assays of In Vitro Cellular Immunity for Monitoring Immunity to Tumor Antigens in Animals and Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James L. McCoy	Expert Consultant	LID	NCI
Others:	R.B. Herberman	Chief, LID	LID	NCI
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	I. Suslov	Visiting Fellow	LID	NCI
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COOPERATING UNITS (if any)

Litton-Bionetics, Inc., Frederick Cancer Research Center, George Washington University, National Naval Medical Center, Walter Reed Medical Center, Portsmouth Naval Hospital, University of South Carolina.

LAB/BRANCH

Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.1	3.1	1.00

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cellular immunity (CI) to tumor-antigens (TA) was evaluated and monitored using microassays of direct and indirect migration inhibition (MI), isotopic footpad and Winn neutralization tests as a function of growth of mouse mammary tumor-virus (MuMTV)-induced tumors, SV-40 virus-induced sarcomas, chemically induced sarcomas and plasmacytomas, and guinea pig hepatocarcinoma. CI to TA and antigens of MuMTV was observed in tumor bearers. CI decreased as magnitude of tumor burden increased. Immunological "eclipse" with plasmacytomas was associated with sephadex G10 adherent cells and the effect was reversed by levamisole therapy. Direct capillary tube and indirect MI microassays demonstrated production of leukocyte inhibitory factor (LIF) of human breast and lung carcinoma, Ewing's sarcoma and melanoma patients in response to tissue culture tumor extracts, and of breast cancer patients to MuMTV. High titered LIF supernatants were generated with small numbers of mononuclear cells by PPD and lung and breast cancer extracts and MuMTV. Physicochemical separation of supernatants were successful in partially purifying LIF. Evaluation of indicator cells for indirect human and mouse MI demonstrated that some humans and mouse strains were insensitive to lymphokine. In mice this was due to suppressor cells.

Project Description:

Objectives: The objectives of this study are (1) to develop and use sensitive and specific *in vitro* micro-assays of migration inhibition, *in vitro* isotopic foot-pad assays in mice and *in vivo* tumor cell neutralization (Winn) assays in mice for detection of cell-mediated immune reactivity to purified protein derivative (PPD) and tumor-associated antigens (TAA) of normal and tumor-bearing hosts in experimental animals and man; (2) monitor antigenic activity of solubilized and partially purified TAA of animal and human tumors; (3) longitudinally monitor host reactivity against TAA, as a function of clinical course of disease in animals on BCG immunotherapy protocols, and in cancer patients to see if migration inhibition assays are predictive of disease recurrence, survival or efficacy of therapy; (4) to measure immunologic cross-reactivity between (a) human tumors of the same or different histologic types; and (b) TAA of human breast cancer and mouse mammary tumor virus (MuMTV) and its major glycoprotein (gp52); (5) to investigate the development with age of natural immunity in C3H mice, and to compare this, in regard to specificity and other characteristics, with the immunity of C3H mice bearing transplantable MuMTV-induced tumors; (6) to study the phenomenon of immunological depression and its restoration by levamisole therapy in mice bearing plasmacytomas or Meth-A sarcomas; (7) analysis of cellular receptors involved in interaction with leukocyte inhibitory factor (LIF); (8) to isolate and characterize LIF; and (9) analysis of mechanisms of unresponsiveness of macrophages of C3H/HeJ mice to MIF.

Methods Employed:

Antigens and lymphoid cells for migration inhibition: Crude or solubilized and partially purified antigen preparations of fresh or tissue cultured tumor and normal cells were made by a variety of procedures including sonication and nitrogen bomb disruption of cells or extraction with 3M KCl and papain and NP-40 detergent digestion. The antigen preparations were quantitated by the protein method of Lowry and aliquotted and stored at -70°C.

Peripheral blood leukocytes, mononuclear cells and polymorphonuclear cells of humans and guinea pigs and peritoneal exudate cells of mice were used.

Migration inhibition assays: Direct capillary tube migration inhibition (LMI) assays were performed with human peripheral blood leukocytes (PBL). Direct agarose microdroplet migration inhibition assays were also run with human or guinea pig PBL or with mouse peritoneal exudate cells. Indirect agarose microdroplet migration inhibition tests were run with LIF produced with human mononuclear cells tested on human indicator polymorphonuclear cells, and migration inhibitory factor (MIF) production by mouse spleen cells tested on mouse peritoneal exudate cells.

Foot Pad Assays in Mice

The ^{125}Iodine isotopic footpad assay as earlier described by (Paranjpe and Boone; J. Natl. Inst., 48:563, 1972) was used to measure immunity against membrane and soluble antigens of SV-40 virus induced tumors and chemically induced Meth A and ADJ-PC5 tumors of mice.

Major Findings:

I. Development and Use of Micro-Migration Inhibition Assays

A. Introduction

The direct capillary tube LMI assay has been used by our laboratory for a number of years to monitor human reactivity against TAA. We have attempted to develop a sensitive direct micro-LMI assay that would require smaller quantities of crude or partially purified antigens and whole blood, as the source of leukocytes. We have developed an agarose microdroplet LMI assay in the human and guinea pig which is less cumbersome than capillary tube assays to routinely perform and which requires only 2-5 ml of whole blood. We have developed indirect migration inhibition assays using indicator human polymorphonuclear cells and mouse peritoneal exudate cells with the agarose microdroplet method. Emphasis was placed on generating supernatants containing high titered LIF and on physicochemically characterizing LIF in these supernatants. We were interested in determining the usefulness of the direct and indirect agarose microdroplet assays for monitoring small quantities of antigen preparations of animal and human tumors that had been solubilized and partially purified.

B. Listed below are summaries of our progress and findings with the agarose microdroplet technique:

1. Human LMI Reactivity in PPD

Direct and indirect agarose microdroplet migration inhibition assays were performed with log dilutions (50 μg-5 x 10^{-8} μg/ml) of soluble purified protein derivative (PPD) of tuberculin and leukocytes (4 x 10^{5}/droplet) from PPD skin test positive or negative individuals. Inhibition of migration of leukocytes from 9/11 PPD skin test positive donors was observed in direct tests with PPD doses of 1-50 μg/ml PPD. Inhibition of migration of leukocytes from some PPD skin reactive donors was obtained with as little as 5 x 10^{-3} -- 5 x 10^{-7} μg/ml (i.e., 5 nanograms to 0.5 picograms). Some inhibition of leukocyte migration was seen with skin test negative donors (presumably toxicity) with the higher doses of PPD (50 μg/ml), but generally little inhibition was observed with these donors at lower doses. Comparative experiments of the agarose micromethod and the capillary tube technique indicated that the agarose assay was more sensitive in that it could detect reactivity with 2-4 logs lower antigen concentrations. Indirect assays using supernatants of cultures of PPD triggered mononuclear cells and indicator granulocytes in agarose droplets demonstrated that the reactions were due to production by immune lymphocytes of a lymphokine (presumably LIF). The results indicate the usefulness of this technique in the sensitive detection of CMI against such antigens as soluble PPD. The assay should prove useful in quantitative assessment of cell-mediated reactivity by using a wide range of antigen concentrations.

2. Guinea Pig LMI Reactivity to PPD and Crude TAA of Line-10 Hepatocarcinoma

Direct agarose microdroplet LMI assays were used to monitor systemic immunity against line 10 extract (TAA) and PPD in a strain 2 guinea pig immunotherapy model. Leukocyte donors were normal animals with progressive intradermal (id) or disseminated tumors or animals whose id tumors received intralesional BCG therapy and surgery, resulting in disease remission or cure. With sensitized animals inhibition of migration was seen with both antigens. Of considerable interest, animals sensitized to only BCG became reactive to TAA while animals sensitized to line 10 reacted to PPD, suggesting common antigens between line 10 and BCG.

Evaluation of reactivity in individual animals did not reveal a predictive pattern in relation to disease prognosis. Some animals destined to die were reactive while other tumor-cured animals responded only weakly or not at all. However, when treatment groups were compared, LMI appeared predictive of prognosis. Groups with large tumor burdens and having a poor prognosis did not respond, as well in LMI against TAA as groups having favorable prognosis. Results emphasize individual variation even with inbred animals, but suggest LMI is predictive of efficacy of therapy on a group basis within randomized protocols.

The LMI reactions appeared to be lymphokine-mediated since similar results were obtained with indirect assays with supernatants generated by interaction of PPD with peripheral mononuclear cells from BCG immunized guinea pigs. The soluble factor in our supernatants was presumably guinea pig leukocyte inhibitory factor (LIF) since the indicator cells were guinea pig polymorphonuclear cells.

3. Monitoring Antigenic Activity of Extracts of Mouse SV40 Virus Induced Tumors, the Mineral Oil Induced ADJ-PCJ Plasmacytoma, and the Methylcholanthrene-Induced Meth-A Tumor.

a. SV-40 Virus-Induced mKSA

Direct macrophage migration inhibition micro-assays, were used to monitor TAA activity of preparations of SV40-induced mKSA cells. The antigen preparations included cell-free crude membranes, papain-solubilized and NP40 detergent-solubilized membrane extracts from mKSA tumor cells. The assay was extremely sensitive and could detect migration inhibition reactivity with all three types of antigenic preparations, with concentrations as low as 250 picograms protein/ml. The reactivities were specific in that peritoneal exudate cells from BALB/c mice, immunized with antigenically unrelated but syngeneic plasmacytomas, were not inhibited by these antigens.

b. ADJ-PC5 Plasmacytoma

Cell mediated immunity, as detected by the direct agarose microdroplet macrophage migration inhibition assay, was investigated with several crude membrane (CM) and solubilized preparations of murine plasmacytomas. Inhibition of macrophage migration was detected with picogram quantities of CM, NP40 detergent- and papain-solubilized preparations (CS) of ADJ-PC5 and LPC-1 plasmacytomas. Specificity studies demonstrated that ADJ-PC5 and LPC-1 plasmacytomas expressed cross-reactive tumor-associated antigens (TAA). No cross-reactivity was observed with similar extracts prepared from the unrelated syngeneic simian virus 40 (SV40)-induced sarcoma. The inhibition of macrophage migration observed was mediated by culture supernatants generated from the mixture of plasmacytoma immune spleen cells with antigens and then assayed in an indirect assay on normal PEC.

c. Meth A Sarcoma

Peritoneal exudate cells (PEC) from BALB/c mice immunized against NP40 detergent preparations of Meth-A tumor cells were used in agarose microdroplet macrophage migration inhibition and Winn tumor cell neutralization assays to evaluate their antigenic specificity. Spleen cells from animals immunized with crude antigen effectively neutralized Meth-A cells in Winn assays. Direct migration inhibition assays were quite sensitive and detected migration inhibition of PE cells from immunized mice using extract concentrations as low as picogram quantities. Specificity studies with the migration inhibition assay demonstrated that Meth-A expressed no antigenic cross-reactivity with similarly prepared extracts of unrelated mKSA sarcoma, or ADJ-PC5 plasmacytoma. Inhibition of PEC migration was mediated by culture supernatants (presumably MIF) generated from the mixture of immune spleen cells with mitomycin treated Meth-A cells as assayed in an indirect migration inhibition test. Mice bearing medium to large-sized subcutaneous Meth-A tumors shown to be paralyzed in Winn assays, nevertheless retained strong reactivity to TAA as measured in the direct migration inhibition assay. The results demonstrate the ability of BALB/c mice to develop CMI to NP40 solubilized preparations of Meth-A tumor cells and further show the ability of animals bearing large tumors to recognize TAA even though they cannot effectively prevent growth of these tumor cells.

5. Monitoring Antigenic Activity of Human Breast Carcinoma and Melanoma Antigen Preparations

We have studied the antigenic activity of crude membrane and partially purified Sephadex G-200 column polyacrylamide gel fractions of the MCF-7 cell line derived from human breast cancer and of melanomas prepared by Dr. Ariel Hollinshead of George Washington University and Dr. James Braatz of LID by the capillary tube and indirect agarose method. Reactivity with patients' leukocytes has been observed with these column and gel fractions with as little as nanogram quantities of materials. Testing by capillary tube LMI of a partially purified Sephadex G-200 column fraction of a human melanoma with a large number of melanoma patients, normal donors and patients with cancer of other sites has revealed that approximately 50% of the melanoma patients react with this G-200 fraction while less than 10% of the normal donors and less than 20% of patients with cancer of other sites are reactive, suggesting that this fraction contains a melanoma TAA. Further testing is in progress to determine the specificity of the reactions with the breast related materials and to determine which fractions may have optimum reactivity.

II. Use of Direct Capillary Tube LMI to Assess Cellular Immunity of Cancer Patients to Human Tumor Antigens

A. Use of direct capillary tube LMI and lymphocyte blastogenesis to measure cross-reactivity between human breast cancer and mouse mammary tumor virus antigens

Because of the potential etiologic and diagnostic implications of a potential specific immunologic relationship between human breast cancers and mouse mammary tumor virus, double blind direct migration inhibition assays and lymphocyte proliferation tests were conducted with leukocytes from breast carcinoma, benign breast disease, other cancer patients, and normal donors using mouse mammary tumor virus (MMTV), its major glycoprotein (gp52), murine leukemia virus (MuLV) glycoproteins (gp69/71), and 2 human glycoproteins and fetuin (a glycoprotein of fetal bovine serum) as antigens. A large proportion (50%) of breast carcinoma patients and some (approximately 30%) benign breast disease patients reacted with MMTV. Normal donors and patients with other cancers were generally unreactive. Migration inhibition with gp52 was positive in approximately 50% of breast carcinoma patients and 25-30% of fibrocystic patients while normal donors and patients with other cancers were unreactive. Preoperative breast cancer patients (65%) were more reactive then patients tested 1 month-2 years post-operatively. There was also a trend for patients developing recurrent disease to have less reactivity then those in the post-operative period having no clinical evident disease. Specificity studies with gp69/71 preparations of Rauscher leukemia and Kirsten sarcoma virus gave approximately 40% incidence of reactivity of breast carcinoma patients, 20% with benign breast disease, 10% with normal donors, and 0% with other cancers. However, significantly more reactivity of breast cancer patients was observed with the 2 human glycoproteins and fetuin than was observed with normal donors or patients with cancer of other sites. Kinetic experiments using different harvest days and population studies with lymphocyte proliferation assays were unable to discriminate breast cancer patients from normal donor reactivity with MMTV, gp52, and gp69/71 antigens. The results suggest that many breast carcinoma and benign breast disease patients have LMI hyper-reactivity against many glycoproteins including those of MMTV and MuLV. Due to lack of restriction of LMI specificity to antigens of MMTV in both patients with malignant and benign breast disease, caution must be presently taken in interpreting the etiologic significance of these findings.

III. Generation of High Concentrations of LIF for Use in Indirect Assays

In addition to developing the direct migration inhibition assay at a more micro-level, we have been interested in developing supernatants to be used in indirect migration tests that contain high titers of LIF. If supernatants containing high concentrations of LIF can be made by small quantities of mononuclear cells, this would help to miniaturize and quantitate the assay.

We have found that supernatants containing high titered LIF (10^{-6} to 10^{-7} dilutions still reactive) can successfully be generated with 2 x 10^7 mononuclear cells from PPD skin-reactive human donors in response to PPD, if the supernatants are made in 50 ml polystyrene concial tubes. Generation of supernatants with only 5 x 10^6 cells in the 50 ml tube or generation in smaller round bottom plastic tubes with 5 x 10^6 or 2 x 10^7 mononuclear cells yields LIF titers that dilute to only 1:2 - 1:10. The results suggest that a combination of the geometry of the tube and the high cell concentration (2 x 10^7) and presumably more cell to cell interaction in a small area is essential for the production of these high titered LIF preparations. During the performance of this study we also observed that some normal donors' polymorphonuclear cells were not sensitive to LIF. The reason is not yet understood, but the finding points out the necessity of testing supernatants with only reactive donor polymorphonuclear cells to eliminate negative tests. Physiochemical studies using Sephadex G-200 columns of these supernatants indicate that the reactive molecule(s) was in the molecular weight range (69,000) of LIF as earlier reported by Rocklin. Further supporting evidence that we are truly dealing with the lymphokine, LIF, in these supernatants was obtained by sugar inhibition and sugar column chromatography studies. Both α L-fucose and N-acetylglucosamine, sugars previously reported to block LIF activity on human polymorphonuclear cells, inhibited the activity of our supernatants, whereas glucose had no inhibitory activity on the LIF containing supernatants. LIF activity could be bound and again removed from α L-fucose and N-acetylglososamine columns, but not from a similarly prepared glucose column. These data suggest that the reactive receptor on the polymorphonuclear cell for LIF has both α L-fucose and N-acetylglucosamine moieties in its structure. Furthermore, these physiochemical studies strongly support the contention that the supernatants do contain high titered LIF.

IV. Indirect Agarose Microdroplet Migration Inhibition Assays to Measure Reactivity of Cancer Patients to Tumor Associated and Other Antigens

A. Breast Cancer Patients

Breast cancer patients, normal donors, and benign breast disease patients were tested in indirect migration inhibition assays with a 3MKCl extract of MCF-7, and with MuMTV or its major glycoprotein, gp52. Initially, supernatants were generated with 5 x 10^6 mononuclear cells, but recently the supernatants have been generated in a micro-system using 5 x 10^5 mononuclear cells (only 5-10 ml whole blood required). The micro-system involves generation of supernatants in 0.4 ml plastic conical microfuge tubes similar in geometry to the 50 cc conical tubes described above for generation of high titered LIF supernatants. More than 50% of the breast cancer patients produced LIF in response to MCF-7, MuMTV or gp52 while no appreciable reactivity was observed when supernatants were generated with the control antigen, murine Rauscher leukemia virus gp70. Approximately 25% of patients with fibrocystic disease and fibroadenoma and less than 10% of the normal donors were capable of producing LIF to MCF-7 or MuMTV. Supernatants generated in the microfuge tubes were reactive in many cases at dilutions of 10^{-4} or 10^{-5}, thus suggesting that semi-quantitative assessment of patient reactivity might be obtained by determining end-point dilutions of the supernatants. Patients with early (Stage 1) as well as those with advanced disease (Stage 3) were able to produce LIF in response to the antigens. As seen with the direct capillary tube testing described above, preoperative patients appeared to be more reactive than those tested after surgery (1 month to >2 years) and having no evident disease.

B. Lung Cancer Patients

Indirect migration inhibition tests with lung cancer patients receiving no radio-therapy or other treatment were tested with 3MKCl extracts of fresh pleural effusion cells from a patient with adenocarcinoma of the lung (7661) and of tissue culture cells (CaLu) derived from a squamous cell carcinoma of the lung. 60-70% of the untreated patients responded, whereas most patients receiving radio-therapy were not capable of producing LIF to these antigens. Of considerable interest, was the observation that many normal donors in close contact with lung cancer patients or with tumor or blood specimens produced LIF in response to these antigens, whereas non-contact donors were unreactive. These studies are being continued under code, with larger numbers of normal donors (both those in contact with patients and non-contact) to determine the validity of this observation. Experiments during the last 6 months were performed with microfuge tubes, using 5×10^5 mononuclear cells to generate LIF. Reactive supernatants could usually be diluted to an endpoint of 1:100 - 1:1000, which may allow for future semiquantitation of patient responsiveness to these antigens.

V. Evaluation of Cellular Immunity to MuMTV Antigens in a Mouse Mammary Tumor Virus Model

Early reports with transplantation rejection studies suggested that C3H (C3H+) mice congentially infected with mammary tumor virus via milk transmisson were immunologically tolerant to antigens of the mammary tumors, whereas C3H (C3H-) mice foster nursed and negative for the virus were immunologically reactive against such antigens. More recently in vitro humoral and cellular immunity against MuMTV antigens have been observed in the high spontaneous mammary tumor strain (C3H+). Thus, the possibility of immunological tolerance to the virus in congenitally or neonatally infected mice has been largely dismissed.

A. Development of Cellular Immunity in Normal and Tumor-Bearing C3H+ and C3H- Mice

We have investigated the development of cellular immunity to MuMTV in normal virgin C3H+ and genetically similar C3H- female mice by the indirect agarose droplet migration inhibition assay. Spleen cells from C3H+ mice were able to respond to MuMTV antigens and produced a factor (presumably macrophage migration inhibition factor, MIF) that inhibited migration of normal mouse peritoneal exudate cells. This reactivity appeared at 14 weeks of age in C3H+ mice but was no longer detectable at 36 weeks. In contrast, reactivity was never displayed by spleen cells of C3H- mice. Injection of 10 μg MuMTV in C3H+ or C3H- mice younger than 14 weeks of age induced similar reactivity to MuMTV antigens in both mouse strains. This pattern of reactivity suggested that normal C3H+ mice are not tolerant to antigens of MuMTV and that overt infection is required for induction of an immune response. Further investigations were performed to see whether the growth of a syngeneic transplantable mammary tumor (MAT-2) affected the migration inhibition response to MuMTV in both C3H+ and C3H- mice. A response to MuMTV antigens could be detected in C3H- mice younger than 14 weeks of age, but C3H+ tumor-bearing mice were generally unable to respond to these antigens. A similar pattern of results in migration inhibition tests with tumor-bearing animals were obtained with gp52, the major glycoprotein of MuMTV, as the antigen. The differences in reactivity of normal, MuMTV immunized and tumor-bearing C3H+ and C3H- mice that have been observed in these studies indicate that development of cell-mediated immunity to MuMTV antigens may be dependent on the dose, route and length of exposure to MuMTV antigens. Furthermore, normal BALB/c mice developed migration inhibition reactivity to MuMTV if they were housed for several weeks in the same room as MuMTV-bearing mice, suggesting that horizontal transmission of the virus is involved in the immunobiology of MuMTV.

B. Development of Cellular Immunity to Tumor-Associated Antigens of Transplantable Mammary Tumors of C3H/HeN Mice

Cellular immunity to tumor-associated antigens of C3H+ and C3H- mice bearing syngeneic transplantable mammary tumors was assessed by the production of MIF in response to MuMTV-related antigens of 3M KCl tumor extracts, using the indirect agarose microdroplet migration inhibition assay. C3H+ mice bearing the MAT-2 transplantable syngeneic mammary tumor were generally unable to produce MIF in response to MuMTV, whereas a strong immune response in those mice was elicited by a 3M KCl extract of MAT-2 tumor. C3H- tumor-bearing mice were reactive to MuMTV as well as to 3M KCl extract of MAT-2. The latter reactivity was found to be specific since these mice failed to react against similarly prepared 3M KCl extracts of a mouse plasmacytoma. Two other spontaneously arising mammary tumors (MAT-3 and MAT-4) of C3H+ mice appeared to share an antigen with MAT-2. Furthermore, a 3M KCl extract of C3H+ embryos, with no detectable MuMTV antigens, was found to stimulate migration inhibition reactivity in C3H+ and C3H- mice bearing MAT-2 tumor, suggesting that tumor growth induces reactivity against non-virus-related TAA which is common to embryo cells. Induction of MIF production by MAT-2 TAA or MuMTV appeared dependent on T cells, since reactivity was eliminated by pretreatment of immune spleen cells with anti-Θ + complement.

C. Investigation of Responsiveness of Genetically Related C3H/Hen and C3H/HeJ to MIF

The migration of PEC from endotoxin-unresponsive C3H/HeJ mice in response to MIF was compared to that of equal numbers of PEC from closely related but endotoxin responsive C3H/HeN mice. Macrophage migration with PEC from C3H/HeN mice was strongly inhibited (less than 50% of control) through a 1/32 dilution of MIF-supernatants. In contrast, no migration inhibition was detected with PEC from C3H/HeJ mice even with undiluted MIF-supernatants. Migration of PEC from C3H/HeN and C3H/HeJ mice in control supernatants or in fresh medium were comparable. Thus, PEC from C3H/HeJ mice were at least 30-fold less responsive to MIF than PEC from C3H/HeN mice. Furthermore, PEC from C3H/HeN mice admixed with 5-10% PEC from C3H/HeJ mice became completely unresponsive to MIF-supernatants. The mechanism for this state of MIF-unresponsiveness and the relationship of this defect to the endotoxin unresponsiveness of C3H/HeJ mice is not yet known. However, comparison of the C3H/HeN and C3H/HeJ strains may provide a useful tool for analysis of macrophage-lymphokine interaction and the modulating effects of bacterial endotoxins. Further, as with the finding that some normal human donor polymorphonuclear cells were unresponsive to LIF, these data indicate that when negative results are obtained in indirect migration inhibition assays, unresponsiveness to the donor's indicator cells must be considered in addition to lack of production of lymphokine.

VI. Use of an Isotopic Footpad Assay for Evalution of Cellular Immunity to Tumor Cells and Solubilized Murine Tumor-Antigens

^{125}Iodine isotopic footpad (IFP) assays were used to measure cell-mediated immune responses _in vivo_ to intact tumor cells, crude membrane (CM) preparations and NP40 solubilized tumor cell extracts in BALB/c mice immunized to the SV40-induced tumor mKSA. A correlation was observed between footpad swelling as measured by calipers and the incorporation of ^{125}I-labelled human serum albumin into footpads of mice challenged with intact tumor cells. The IFP measurements were favored over footpad swelling measurements since the former lack the subjectivity involved in measuring footpad thickness and appear more sensitive and rapid to perform. These studies indicate that tumor-associated antigens on intact tumor cells from SV40-induced tumors can be recognized _in vivo_ in the IFP assays and the response is specific to the sensitizing tumor in that other syngeneic tumors do not cross-react. In addition, these data represent the initial success in using solubilized TAA in the IFP assay. Reactivity to TAA on either CM or NP40 crude soluble extracts of mKSA TC were observed, indicating that the required structural integrity for immune recognition of TAA was preserved following cell disruption and solubilization. Thus, NP40 represents a good method of obtaining solubilized TAA from TC membranes. Local adoptive transfer of CMI reactivity to mKSA TC in the IFP assay was achieved with spleen cells, but not serum, from hyperimmune mice, further implicating the cell-mediated nature of this _in vivo_ response. Finally, the IFP assay appears to be a rapid, reproducible and sensitive method of measuring cellular immune reactivity to TAA on either intact tumor cells or solubilized from the cell membrane by detergent.

VII. Investigations of Depression of Immune Competence in Mice Bearing Plasmacytomas and its Reversal by Levamisole Therapy

General and tumor-specific cell-mediated immunity was investigated in BALB/c mice bearing progressively growing mineral oil-induced plasmacytoma ADJ-PC5, which was previously known to possess tumor-associated antigens (TAA). Lymphocyte blastogenesis (LB) assays demonstrated that the levels of blastogenesis to T- and B-cell mitogens in spleen cells of tumor-bearing mice were severly depressed in the early as well as the late stages of tumor growth. Total eradication of the palpable tumor after chemotherapy with aniline mustard resulted in recovery of the general T- and B-cell immunocompetence. Spleen cells from mice bearing medium-sized to large (15-30 mm in diameter) subcutaneous ADJ-PC5 tumors, shown to be generally depressed to mitogens by LB assays, nevertheless retained some tumor-specific neutralizing immunity in Winn assays against ADJ-PC5 tumor cells. Specificity of the ADJ-PC5-directed immunity was confirmed by the failure of ADJ-PC5-immune spleen cells to neutralize a Moloney virus-induced leukemia (LSTRA) or a simian virus 40-induced fibrosarcoma (mKSA). Likewise, established specific immunity against TAA of mKSA was not interrupted by progressive growth of ADJ-PC5 tumors in mKSA-immune mice. Results suggested that specific cell-mediated immunity against TAA of ADJ-PC5 or another tumor (mKSA) was left intact in animals bearing progressively growing ADJ-PC5 tumors, even though their spleen cell populations (T and B) demonstrated severly depressed lymphoproliferative responses to T- and B-cell mitogens. The depression appeared to be due to Sephadex G-10 column adherent cells (presumably a macrophage), since LB responses were restored following the removal of these cells and since the addition of these cells to normal spleen cells resulted in suppressed responses.

In vivo restorative effects of Levamisole on the general and tumor-associated cell-mediated immune responses were then investigated following aniline mustard chemotherapy of mice bearing the ADJ-PC5 plasmacytomas. Levamisole was employed following aniline mustard chemotherapy in an attempt to restore immunocompetency. Administration of Levamisole following drug treatment had a significant effect on the ability of mice to resist rechallenge with ADJ-PC5 tumor and in Winn tumor cell neutralization. The enhanced resistance to tumor cell challenge appeared to be associated with a faster recovery of the general T-cell immunocompetence as demonstrated in LB assays among the mice receiving chemotherapy followed by adjuvant therapy. Levamisole, when administered alone to tumor-bearing mice, did not appear to possess a direct antitumor effect. In addition, Levamisole did not potentiate cellular immunity to higher than normal levels in nonimmunodepressed mice. These results demonstrated the efficacy of Levamisole as a restorative agent of the general and tumor-associated immunocompetence in the immunodepressed host, possibly by abrogating effects of supressor cells.

Significance of Research:

The ability to successfully develop direct and indirect migration inhibition microassays for measuring cellular immunity against tumor-associated antigens is of considerable interest and importance. This should permit the possible application of this assay for diagnosis of cancer or for monitoring disease progression or regression following treatment. This would fall under Objective IV, Approach 4 of the National Cancer Plan.

Similarly, it is important in longitudinal patient monitoring to have standard batches of antigen sources available for repeat testing. This has been provided by tissue culture cells derived from breast cancer, lung cancer and Ewing's sarcoma. Such cultured cells, since they do possess antigenicity, are excellent candidates to be used for TAA isolation and purification. The migration inhibition assay appears to be quite useful to monitor the purification of human tumor-associated antigens from these cells. This would fall under Objective VI, approach 1, 2 and 4 of the National Cancer Plan.

Detection of cellular immune reactivity of breast cancer patients to antigens of mouse mammary tumor virus may be valuable for the diagnosis and monitoring of breast cancer patients. Further, such cross-reactivity may ultimately shed light on the etiology of human breast cancer. This would fall under Objective V, Approach 4 of the National Cancer Plan.

Demonstration of apparent cell-mediated immune cross-reactivity between antigens of BCG and line 10 hepatocarcinoma cells may help to explain the mechanism of the immunotherapeutic activity of BCG, at least in this animal model system. Further, the longitudinal monitoring of immunity to BCG and TAA of line 10 tumors by migration inhibition during the course of a well-controlled immunotherapy trial may help provide information about the usefulness of the migration inhibition assay for predicting prognosis or response to therapy.

The utilization of murine mammary virus-induced, SV-40 virus-induced and chemically-induced tumor models to investigate the development of immunity against TAA and mammary tumor virus antigens as a function of progressive tumor growth may provide insights into the use of such assays.

Future Studies:

We will continue to develop indirect micro-assays of migration inhibition to be used for longitudinal monitoring of breast and lung carcinoma patients with 3MKCl extracts of tissue culture derived cell lines. Emphasis will be placed on generating high titered LIF supernatants which may allow for semi-quantitation of the assay by titrating the supernatants. Further, breast cancer patients will be monitored by these micro-assays with mouse mammary tumor-virus and gp52. These assays will also be used to monitor physicochemical separation and partial purification of tumor-associated antigens of human breast and lung carcinomas.

Investigations concerning the physicochemical characterization of human LIF in supernatants generated with PPD and human tumor-antigens will be continued by column chromatography and LIF-inhibition studies with known inactivators of the lymphokine including specific sugars and anti-esterases.

Recently, others in our laboratory have demonstrated a requirement of histocompatible macrophages in populations of rodent lymphoid cells used to generate MIF by soluble antigens. We will investigate whether autologous macrophages are essential in the generation of human LIF in response to PPD and solubilized tumor-antigens. Studies will also be performed to determine whether LIF and MIF generated in one species (e.g., human) will inhibit indicator migrating cells of another species (e.g., mouse and guinea pig).

Studies with the line 10 tumor guinea pig immunotherapy model immunizing intradermally with a vaccine prepared from cryopreserved and irradiated line 10 cells mixed with BCG have recently demonstrated protection and cure of systemic line 10 metatastes. We will monitor this new immunotherapy regimen in direct LMI assays with PPD, in attempts to determine whether the test can be predictive of disease recurrence and survival.

Investigations of host CMI responses against mouse mammary tumor-virus, gp52 and a variety of other related and unrelated antigens were performed using migration inhibition assays and lymphoid cells of normal and mammary tumor-bearing C3H/HeN mice during the past year. We will continue to evaluate the specificity of these reactions in detail and will attempt to determine the mechanism(s) responsible for the immunological "eclipse" as a function of progressive tumor growth observed in those studies. We will monitor migration inhibition reactivity against these antigens as a function of chemotherapy-induced tumor regression and disease recurrence, to see if we can predict which animals will remain tumor-free and which will relapse.

We will investigate the mechanisms of unresponsiveness to MIF of the macrophages of C3H/HeJ mice and some other mouse strains. Preliminary results suggest that supressor cells are involved in the refractiveness to the lymphokine.

Studies will be initiated with a mouse lung tumor (M-109) model to evaluate:

a. its antigenicity and the cellular immune responses in immunized and tumor-bearing hosts;
b. determine whether the migration inhibition assay can predict disease recurrence in tumor-bearing animals undergoing surgery and chemo therapy-induced disease remission;
c. see if immunostimulators (e.g., Levamisole, BCG, pyran, poly I:C, thymosine) are helpful in inducing disease remission or causing a more rapid recovery of immunocompetence following immunodepression by chemotherapy treatment.

Studies related to the mechanism(s) of general immunodepression and immunological restoration in animals bearing mouse plasmacytomas and methylcholanthrene-induced tumors (e.g., Meth A) will be continued. Emphasis will be placed on evaluating the role of suppressor cells and the mechanism of how they contribute to general immune depression.

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LABORATORY OF IMMUNOBIOLOGY

SUMMARY REPORT

October 1, 1977 to September 30, 1978

INTRODUCTION

The Laboratory of Immunobiology, in its second year as a component of the Division of Cancer Biology and Diagnosis, still consists of three sections: Cellular Immunity, Humoral Immunity and Immunopathology. The members of these sections, through their studies of tumor immunology, continue to make significant contributions to the goals of the National Cancer Program. Methods are sought to kill tumor cells in vitro, to eradicate tumors and metastases in vivo and to overcome the possible suppression of host immunity by tumors. In the Cellular Immunity Section a guinea pig hepatoma model has been used to find immunological methods of dealing with malignant disease remaining after surgery. Immunization, after surgery with tumor cells together with living BCG has prevented the progression of residual disease. The effectiveness of immunization in eradicating disease remaining after surgery has been improved by the use of emulsions containing fractions of mycobacteria rather than the living organisms. Factors influencing the development of tumor immunity after surgery alone have been found to include the number of tumor cells initially implanted, tumor mass at the time of surgery, time between tumor implantation and surgery and extent of surgery. A major advance has been made with the finding that intralesionally injected BCG cell walls incorporated into mineral oil droplets were at least as effective as living BCG in eradicating tumors and microscopic lymph node metastases but the non-living preparation lacked the toxic side effects of the living organisms. In studies on the effectiveness of intralesionally injected BCG cell walls to eliminate distant metastases, it was found that tumor cells injected intradermally and contralaterally on the day of treatment were eradicated. A model for head and neck cancer was developed; tumor cells injected into the buccal pads of guinea pigs grew, metastasized and could be eradicated by intratumoral BCG cell walls. Various components of mycobacteria and other organisms are being tested for anti-tumor activity in mice and guinea pigs. BCG cell walls, cell wall skeletons, MER, cord factor, a synthetic analog of cord factor and sulfolipids each have anti-tumor activity when injected intralesionally in the form of an oil in water emulsion. Factors that influence the potency of these agents to cause tumor regression are now well enough understood to anticipate the possibility that a defined preparation containing completely synthetic or synthesizable components will soon be available for experimental immunotherapy. In a study of naturally occurring bovine squamous cell carcinoma it has been found that the efficacy of intralesionally injected BCG cell wall emulsions was approximately equivalent to local surgery in controlling the disease. Mice with a genetic defect in their ability to respond to endotoxin required more living BCG to suppress tumor growth than responder mice. Studies on the in vitro sensitization of guinea pig lymphocytes to tumor antigens are being discontinued.

In the Humoral Immunity Section studies are continuing on the relationship between lipid synthesis by tumor cells and their susceptibility to killing by antibody and complement. Results of several lines of investigation support the conclusion that lipid synthesis (especially of cardiolipin and triglycerides) is responsible for the ability of tumor cells to resist killing by antibody and complement. Molecular formulas have been determined for complexes formed between Protein A and IgG antibodies; the largest of these complexes behaved in certain respects like IgM. These fundamental studies have led to the development of a general immunoassay method that circumvented the need for labelled antigen or antibody. The plant lectin concanavalin-A interacted with IgM antibodies specific for Forssman antigen and was also capable of inhibiting the hemolytic activity of the first and second components of complement. Protein A and some plant lectins are being used as probes to study the molecular basis of the functional activites of antibodies and complement components. In the Immunopathology Section lymphocyte derived factors (lymphokines) that are required for the activation of macrophages are being characterized biochemically. Current evidence indicates that the precursors of macrophages activated by lymphokines are immature blood-derived mononuclear phagocytes. Mice genetically incapable of developing tumoricidal macrophages in response to certain stimuli are being used in studies aimed at elucidating the pathways for macrophage activation. Macrophages from mice with tumors have depressed chemotactic responses and enhanced phagocytosis. Enhanced phagocytosis was transferrable to normal mice by serum. A serum factor, macrophage stimulating protein (MSP), which was found to promote chemotaxis by certain stimuli is being characterized biochemically. Lymphotoxin assay in vitro continues to suffer from a variety of puzzling complexities. A factor was isolated from phytohemagglutinin which increased basophil chemotaxis response to a complement-derived or a leukocyte-derived chemotactic factor.

I. CELL MEDIATED KILLING OF TUMORS

A. Immunotherapy of minimal residual malignant disease. Efforts to develop reliable procedures for the eradication of minimal residual malignant disease have continued. Guinea pigs with established dermal tumors and microscopic metastases in the superficial distal axillary (SDA) lymph nodes were treated by excision of dermal tumors followed by immunization with various vaccines with or without added tumor cells. Intradermal injection of living BCG between the site of tumor excision and SDA lymph node led to significant prolongation of survival in 2 of 5 experiments. The survival of guinea pigs treated by tumor excision and contralateral intradermal injection of living BCG was similar to that of animals treated by tumor excision alone. Vaccines containing both tumor cells and living BCG were not more effective in preventing growth of metastases than vaccines containing BCG alone. Nonliving bacterial vaccines were evaluated in 4 experiments. BCG cell walls (750-900 μg) or a mixture containing BCG cell wall skeleton (CWS, 300 μg), endotoxin (300 μg) and cord factor (150 μg) administered intradermally adjacent to the site of excision prolonged survival. Contralateral injection of BCG cell walls or CWS + endotoxin + cord factor had no antitumor effect. Vaccines containing nonliving microbial adjuvants (either BCG cell walls or BCG CW + endotoxin + P3) mixed with tumor cells were more effective in preventing growth of microscopic lymph node metastases than were vaccines containing adjuvants alone. Ipsilateral or contralateral intradermal injection of vaccines containing adjuvants and tumor cells led to prevention of growth of lymph node metastases in a significant number of animals. These results suggest that it may be possible to develop procedures effective in eradication of malignant disease that may remain after surgery or other currently accepted treatments.

B. Surgery and immunity. Previous studies demonstrated that animals with stage I disease cured by radical surgery failed to develop resistance to growth of an intradermal challenge. If radical surgery was delayed until the disease reached stage II, resistance to contralateral challenge developed. Recent experiments evaluated the influence of tumor dose, tumor burden and timing of surgery on the development of resistance to tumor challenge. The time of appearance of host resistance to tumor challenge was a function of the number of cells in the initial inoculation. Resistance to tumor challenge in animals that received an initial implant of 10^5 tumor cells appeared one week later than in animals that received 10^6 cells. Development of resistance to tumor challenge required the persistence of the dermal tumor for 21 days. Animals treated by removal of the primary tumor 7 days after tumor implantation followed by removal of the node metastases 21 days after implantation failed to reject a challenge with 10^6 viable line 10 cells. Animals treated by radical surgery to remove the primary tumor and node metastases 21 days after initial tumor implantation had strong resistance to tumor challenge.

Attempts to detect concomitant immunity to line 10 tumor were unsuccessful. However, significant levels of transplantation resistance were detectable when contralateral intradermal line 10 tumor was given immediately after surgery. Failure of systemic tumor immunity to develop in guinea pigs after radical surgery during early stage I disease may have implications for the surgical management of human cancer. Surgery for human cancer at an analagous disease stage might leave the patient with occult metastases and no tumor immunity unless immunotherapy is part of the treatment regimen.

C. Intralesional immunotherapy. A BCG cell wall vaccine with improved antitumor activity was developed. This vaccine contains increased amounts of mineral oil and cell walls. The safety and efficacy of this nonliving vaccine was compared to the safety and efficacy of living BCG. Treatments with various doses of therapeutic agents were given at different times after tumor implantation to test the efficacy of each preparation in the treatment of animals with malignant disease. The lowest dose of living BCG used produced a significant cure rate and no grossly evident toxicity. An intermediate and the highest dose of living BCG used cured some animals but others lost weight and a significant number died as a result of the treatment. Histologic examination of animals with significant weight loss showed fatty degeneration of the liver, granulomatous hepatitis and histiocytic infiltration of the spleen. None of the doses of BCG CW used was toxic and all were at least as effective as living BCG in intralesional treatment. In some experiments in which treatment was delayed it was found that the extent of disease required to render treatment ineffective was about the same for living BCG and for BCG CW. These results support suggestions that certain cancer patients should be treated by intralesional injection of BCG CW. BCG CW were effective in producing tumor regression, produced no systemic toxicity and augmented the development of tumor transplantation immunity.

It has been reported that intralesional injection of living BCG augmented the ability of the host to prevent the growth of intravenously injected tumor cells; excision of the primary tumors and regional lymph nodes was required to prevent progressive growth of regional malignant disease. Experiments were performed to determine whether intralesional injection of BCG CW vaccines would augment the ability of the host to reject distant microscopic metastases. Intralesional injection of BCG CW enabled treated animals to eliminate 10^5 tumor cells in the skin of the contralateral flank. Treated animals eliminated 100 times more tumor cells than untreated control animals. These results confirm previous observations that in the line 10 model intralesional treatment enables the host to reject distant foci of metastastic disease. Further experiments are underway to more completely define the requirements for elimination of systemic metastatic disease by immunotherapy.

As part of a program to study intralesional therapy for patients with squamous cell carcinoma of the head and neck, we developed an animal model to evaluate the effects of immunotherapy on tumors growing in the mouth. Oral tumors with associated cervical lymph node metastases developed after injection of tumor cells into buccal pads of inbred guinea pigs. Intralesional injection of living BCG or BCG cell walls caused regression of established tumors, prevented the development of cervical lymph node metastases and led to the development of host resistance to the growth of a subsequent tumor transplant. Results of these experiments support the safety and validity of attempts to treat selected patients with squamous cell carcinoma of head and neck by intralesional injection of BCG CW.

Studies were undertaken to compare the efficacy of BCG cell wall and cell wall skeleton (SK) vaccines in producing regression of the line 10 guinea pig hepatoma and their immunogenicity as judged by delayed cutaneous hypersensitivity. On a weight basis, SK were as effective as CW in causing tumor regression and they provoked delayed cutaneous hypersensitivity reactions in animals immunized with CW or with SK. On a weight basis, CW were more active than SK in eliciting delayed cutaneous hypersensitivity reactions in sensitized guinea pigs whether the animals were immunized with CW or with SK. These results provided no compelling reasons for using SK instead of CW in clinical trials of cancer treatment by mycobacterial vaccines.

D. Basis of antitumor activity of living BCG and of mycobacterial components. Present data have suggested that tumor rejection mediated by BCG required lymphocytes sensitized to mycobacterial antigens, lymphokines, activated (tumoridical) macrophages and perhaps lymphocytes sensitized to tumor antigens. Better understanding of this process might come from studies in animals with inherited disorders in one or more of these components of the immune response. For this reason we studied tumor suppression by BCG in C3H/HeN and C3H/HeJ mice. These two mouse strains were histocompatible and appeared to differ at the *Lps* gene locus. C3H/HeN mice were $\underline{\text{Lps}}^{n}$ (normal) and responded to endotoxin; C3H/HeJ mice were $\underline{\text{Lps}}^{d}$ (defective) and had reduced responses to endotoxin. C3H/HeJ mice, unlike C3H/HeN mice failed to produce tumoricidal macrophages after intraperitoneal infection with living BCG. Experiments indicated that significantly more BCG was required to suppress tumor growth in endotoxin unresponsive mice (C3H/HeJ) than in endotoxin responsive mice (C3H/HeN). The correlation between endotoxin responsiveness, ability to produce activated macrophages and ability to inhibit tumor growth at sites of BCG infection suggested that traits controlled by the Lps gene had a major influence on ability of the host to kill tumor cells. The fact that at sufficiently high concentrations BCG inhibited tumor growth in C3H/HeJ mice suggested that either alternative pathways of tumor cell destruction not involving activated macrophages exist in these mice or that under certain circumstances in vivo, they may produce tumoricidal macrophages.

A recent report suggested that conditions optimal for suppressing the growth of tumor cells in BCG-tumor cell mixtures were detrimental to the development of systemic tumor rejection immunity. Experiments were performed with a transplantable murine fibrosarcoma to evaluate the generality of this observation. Syngeneic mice received intradermal injections of tumor cells and living BCG. The vaccine site was excised 8 days after tumor implantation to prevent possible progressive tumor growth. Animals were challenged 16 days after vaccine injection with a LD(100) dose of tumor cells. The dose of BCG required to suppress tumor growth correlated well with the dose of BCG required to augment the host immune response to tumor. Optimal induction of tumor immunity required injection of 10^6 tumor cells admixed with 2×10^7 BCG. The minimum number of tumor cells required for immunization was 10^5 cells; the minimum number of BCG required for adjuvant activity was 2×10^6 microorganisms. These results did not confirm the observations of others who studied the effects of BCG infection on growth of and host resistance to the LSTRA Moloney virus-induced leukemia.

Intralesional injection of an emulsion containing trehalose dimycolate (TDM) and mineral oil was highly effective in causing regression of established murine fibrosarcomas. Experiments were performed to determine whether tumor regression after intralesional injection of TDM required an immunologically competent host. Tumors of intact and thymectomized irradiated mice were treated by intralesional injection of TDM. Tumor incidence in thymectomized irradiated mice treated with TDM was not significantly different from tumor incidence in intact mice treated with TDM ($p = 0.06$, Fisher Exact Test).

E. Immunotherapy with defined bacterial components. Experiments were performed to isolate and define mycobacterial components with maximal antitumor effects and minimal toxicity. The major findings were:

Injection of emulsified BCG cell walls or MER into transplants of an established, syngeneic murine fibrosarcoma induced complete regression of tumor in a number of mice. The ability of CW or MER to cause tumor regression depended on the amount of oil in the emulsion.

Emulsions of mycobacterial sulfolipid I or III in admixture with endotoxin were able to cause regression of a transplanted guinea pig hepatoma. Emulsion of sulfolipid I or III were not granulomagenic nor toxic after iv administration into mice, and were ineffective in causing regression of established murine fibrosarcoma.

Injection of emulsified trehalose-6,6'-dipalmityl palmitate (TDPP), a synthetic analog of the mycobacterial glycolipid trehalose-6,6'-dimycolate (TDM) into transplants of an established syngeneic murine fibrosarcoma induced complete regression of tumor in a number of animals. The ability of TDPP to cause tumor regression depended on the amount of oil in the emulsion. Emulsions of TDPP were less toxic and less granulomagenic in mice when compared to TDM. Emulsified TDPP in admixture with endotoxin caused regression of a transplanted guinea pig hepatoma. Neither emulsified endotoxin alone nor emulsified TDPP alone were able to cause regression of the guinea pig hepatoma.

Intralesional treatment of transplants of established, syngeneic murine fibrosarcomas with emulsified TDM was compared to treatment with endotoxin or emulsified turpentine. Tumors regressed completely after the treatment with these agents. The histological changes in the injected sites induced by these agents were similar: local necrosis and marked infiltration with neutrophils which was followed by mononuclear cell infiltrate and reparative fibrous tissue. In the regional lymph nodes depletion of the paracortical area and histocytosis were evident after the treatment with TDM or turpentine. Treatment with endotoxin induced increase in size of the regional lymph nodes and lymphocyte proliferation occurred in the B cell dependent areas. No granulomas were found in the injected site or in the draining lymph nodes after the treatment with TDM. Results of this study do not lend support to the hypothesis that granulomagenic activity is required for anti-tumor action of TDM.

A single intralesional injection of emulsified croton oil into transplants of an established, syngeneic murine fibrosarcoma or guinea pig hepatoma caused complete regression of the primary tumors. In guinea pigs the development of the palpable lymph node metastases was delayed but the guinea pigs died of progressive tumor growth.

II. COMPLEMENT MEDIATED KILLING OF TUMORS

A. Modulation of cell susceptibility to killing. Antibody-sensitized line 10 guinea pig tumor cells are resistant to lysis by guinea pig complement (GPC). These antibody-sensitized tumor cells treated with GPC (TAC) incorporated (into complex cellular lipids) and released increased amounts of fatty acids within 5 to 10 min after the addition of GPC as compared to control cells. Treatment of the cells with antibody alone, GPC alone, or antibody plus heat-inactivated GPC did not result in enhanced synthesis or release of lipids. No enhancement in DNA, RNA, or protein synthesis in TAC was noted. Line 1 cells, which can be killed by GPC when sensitized with excess anti-Forssman IgM antibody, demonstrated enhanced lipid synthesis within 1 to 3 min after the addition of GPC to the antibody-sensitized cells, before measurable killing of the cells had occurred. This effect persisted in the surviving cells when tested 5 and 10 min after the formation of TAC. The amount of lipids synthesized and released in these cells was dependent on the amount of antibody used to sensitize the cells. Under conditions where the cells were not killed (i.e., for line 10 tumor cells), the amount of lipids synthesized and released was not dependent on the antibody concentration used to sensitize the cells. For both line 1 and line 10 cells, the effect on lipid synthesis was dependent on the concentration of GPC used. Addition of GPC deficient in C4 to antibody-sensitized cells did not result in enhanced lipid synthesis or release. These data suggest that the synthesis of macromolecules of which lipids are a major component is of central importance for the ability of the cells to resist antibody-GPC mediated attack.

In recent experiments attempts were made to identify the lipids that were involved in the interaction between cells and complement. Since certain metabolic inhibitors or chemotherapeutic agents increase the susceptibility of line 1 or line 10 guinea pig hepatoma cells to humoral immune attack, they were studied for their effects on the ability of the cells to synthesize lipids. A direct correlation was found between the drug-induced increase in sensitivity to antibody-C mediated killing and the inhibition of the ability of the cells to incorporate acetate, glycerol, and fatty acids into complex cellular lipids. Drug-treated cells recultured in drug-free medium regained their resistance to antibody-C mediated killing; these cells recovered their ability for complex lipid synthesis at this time. Thin layer chromatography of $CHCl_3:CH_3OH$ lipid extracts from these cells indicated that the drug-induced increase in susceptibility to humoral immune attack correlated with the inhibition of acetate, glycerol, and fatty acid incorporation into cardiolipin and triglyceride in line 10 cells and the inhibition of incorporation of these compounds into cardiolipin alone in line 1 cells. No direct correlation was found between the sensitivity of the cells to humoral immune attack and the ability of the cells to incorporate precursors of lipid synthesis into other lipid moieties (sphyngomyelin, phosphatidyl serine, phosphatidyl choline, phosphatidyl glycerol, or cholesterol esters). The synthesis of cardiolipin and triglycerides, therefore, appears to be associated with the mechanism whereby these tumor cells resist antibody-C mediated killing.

B. *Interation of Protein A and immunoglobulin*. To gain better understanding of the mechanisms of action of antibody and complement, investigations were continued at the molecular level into the nature of interaction between immunoglobulins, complement components, and antigens with Protein A and with several plant lectins. Results demonstrated that Protein A reacted specifically with IgG present in either whole serum or with IgG bound to its specific antigen on the surface of erythrocytes or tumor cells. Protein A interacted efficiently with human, guinea pig and rabbit IgG. The molecular composition of PA-IgG complexes was studied extensively. Evidence indicates that the largest complexes found had the molecular formula $[(PA(IgG)_2]_2$ although other, smaller complexes could also be found depending on the ratio of IgG to PA. $[PA(IgG)_2]_2$ complexes behaved like IgM molecules with respect to their ability to fix C1 and initiate cell lysis. Although PA did not inhibit the ability of IgG antibody to bind to its specific antigen, complement fixing activity could be enhanced, inhibited or unaffected depending on the ratio of PA to IgG. Since all complexes were formed randomly, a PA-IgG complex was composed of IgG molecules of differing antigenic specificity.

As an outgrowth of these fundamental studies a general immunoassay method was devised which circumvented the need for labelled antigen or antibody.

C. Interaction of plant lectins and complement. While PA interacted only with IgG, the picture was much more complex with the various plant lectins tested. Concanavalin A (Con A), either in solution or insolubilized by covalent binding to Sepharose 4B, inhibited the ability of fluid phase IgM but not IgG anti-Forssman antibody to sensitize sheep red cells (E) toward lysis by excess guinea pig complement. The efficiency of IgM antibody was unaffected when E were treated with Con A before sensitization or when antibody sensitized cells (EA) were exposed to the lectin and washed before the addition of complement. Con A also inhibited the hemolytic activity of guinea pig complement.

The degree of inhibition observed for a given quantity of GPC was dependent on the Con A concentration. Specifically, Con A inhibited the activity of the early acting complement components C1 and C2 in the fluid phase, but had no significant effect on lysis once these components were bound to EA. Inhibition of C2 activity resulted from a direct interaction between Con A and C2 and not from a decreased number of effective EAC14 sites. Con A and C2 apparently did not compete for the same binding site on the indicator cells. The lectin had no observable effect on either fluid phase or cell-bound C4 activity. Under similar conditions, wheat germ or soy bean agglutinin, leucoagglutinin or pokeweed mitogen did not inhibit hemolysis.

Con A was also found to inhibit the killing of antibody-sensitized line 1 tumor cells (TA) by GPC but not by human complement (HuC). Other plant lectins (wheat germ, leucoagglutinin, and pokeweed mitogen) were also tested but Con A was the only lectin found to inhibit antibody-GPC-mediated killing. The inhibitory effect of Con A was observed when the GPC was mixed with Con A or when the antibody-sensitized cells were pretreated with Con A (TA-Con A) before the addition of GPC. The effect could be reversed by treatment of such cells with α-D-methylglucopyranoside or by incubation at 37°C for approximately 2 hr. Con A appeared to act by preventing the binding of the first component of GPC to antibody-sensitized tumor cells. Differences in the binding of the first component of HuC (HuC1) and GPC1 to TA-Con A suggested that a difference in the binding site for HuC1 and GPC1 might exist. It is, therefore, apparent that PA and some plant lectins serve as molecular probes for correlating structural and biological activity of immunoglobulin and C components; experiments are underway in this direction.

III. IMMUNOLOGICAL INTERACTIONS OF HOST AND TUMOR

A. Macrophages. The progression of neoplastic disease depends on complex interactions between host and tumor. Mononuclear phagocytes play a central role in these interactions: macrophages have potent antitumor actions; tumor has profound effects on mononuclear phagocyte function. Macrophages with direct tumoricidal capacity, defined as activated macrophages, develop during a variety of immune reactions including tumor rejection. Development of activated macrophages requires the presence of an effective activation stimulus acting on subpopulations of mononuclear phagocytes responsive to the stimulus:

1. Macrophage activation factors are released from sensitized lymphocytes on contact with specific antigen. These factors are destroyed by heat (56°C for 30 minutes), by extremes of pH (below 4 and above 10) and by proteolytic enzymes. Chromatography of lymphokine-rich supernatants on the basis of size and charge suggests a limited size heterogeneity at a molecular weight of about 55,000 and an extensive charge heterogeneity suggesting the existence of more than one macrophage activation factor.

2. Quantitative analysis of lymphokine-induced macrophage activation showed that not all macrophages could be activated. Tumor cytotoxicity by peritoneal exudate macrophages activated in vitro by lymphokines was about 10-fold greater than that of equal numbers of lymphokine-treated resident peritoneal cells. This was due to an increased number of lymphokine-responsive cells in acute inflammation. They were peroxidase-positive and represented an influx of mononuclear phagocytes from the peripheral blood. The studies suggested that the lymphokine-responsive precursor of tumoricidal macrophages during both steady state conditions and acute inflammation was the immature blood-derived mononuclear phagocytes.

In certain situations, an activation stimulus acting on responsive mononuclear phagocytes was not sufficient for development of tumoricidal activity. C3H/HeJ mice, genetically unresponsive to the biological effects of bacterial lipopolysaccharide (unlike responsive C3H/HeN mice), failed to develop tumoricidal macrophages after intraperitoneal injection of viable M. bovis, strain BCG. Macrophages from these mice also did not become cytotoxic after in vitro treatment with macrophage activation factors present in lymphokine supernatants. The macrophage tumoricidal defect of C3H/HeJ mice is highly restricted: inflammatory responses to BCG infection in vivo or macrophage responses to chemotactic lymphokines in vitro were normal. Furthermore, production of macrophage activation factors by C3H/HeJ lymphocytes was normal. Further studies of these strains should throw additional light on the pathways for macrophage activation.

Macrophage function in mice with transplanted syngeneic fibrosarcomas was examined with 2 in vitro assays, chemotaxis and phagocytosis. In contrast to the previously reported persistent depression of chemotactic responses of resident peritoneal macrophages from these mice, phagocytosis of antibody-coated erythrocytes was markedly increased. Phagocytic capacity was 4-5 times normal within 1 week of tumor transplantation and returned to normal by 2 weeks. The increased phagocytic capacity, but not the chemotactic defect, could be transferred to normal mice by serum. The serum activity did not necessarily correlate with phagocytic capacity of the tumor-bearing donor: serum factors were detected for 6 weeks after tumor transplantation even at times when donor mice had normal phagocytosis.

It appears likely that physiological control of the mononuclear phagocyte system is mediated in part by humoral factors. One such factor, macrophage stimulating protein (MSP) was found in the serum of all mammals tested. It was assayed by its capacity to promote chemotaxis of mouse resident peritoneal macrophages to chemotactic stimuli such as endotoxin activated mouse serum. MSP itself is not a chemotactic stimulus. Further work on the characterization of this protein included development of a large scale fractionation schema suitable for 3 liters of human plasma, purification of MSP antigen approximately 2500-fold and production in rabbits of antibody that neutralizes MSP activity. Immunoabsorbent columns were made by reacting anti-MSP antiserum with Protein-A-Sepharose. In addition to its chemotaxis-promoting effect, the MSP fraction also increased phagocytic activity and caused formation of long cytoplasmic processes. All 3 actions were abolished by anti-MSP antiserum. These 3 cellular activities involve movement of at least a portion of the macrophage and it is likely that the MSP fraction stimulates a rate-limiting step in a movement or contractility pathway. A potent SH compound, dithiothreitol, eliminated MSP-induced cell process formation and phagocytosis stimulation (effects on chemotaxis were not tested) but had no effect on unstimulated phagocytosis. DTT did not affect MSP binding. The results suggest that a disulfide compound plays a role in the MSP stimulationg pathway.

B. Lymphotoxin. The release of tritium from thymidine-labeled target cells has been used as an assay of both cell and factor-mediated cytotoxicity. This assay is complicated by several findings: (1) The release of tritium is inhibited by undiluted cytotoxic culture fluids from stimulated spleen cells, despite visual evidence of extensive target cell destruction. Tritium release is also inhibited by sonicates of target cells and by concentrated conditioned medium from some lymphoid cell lines. (2) The tritium release is increased by incubation of the cells with DNase or trypsin. This occurred even when the enzyme was added for a 15 minute period at the end of the assay. Despite a large release of ^3H, there was no significant change in the number of target cells. Furthermore, by dye staining criteria, the remaining target cells before and after the 15 minute DNase treatment were viable and contained DNA. This puzzling finding is probably best explained by release from dead cells of not only soluble ^3H but also ^3H in compounds that adhere in an insoluble state to tissue culture plastic or viable cell surfaces until acted on by DNase or trypsin.

Factors that cause tritium release from target cells were produced by established cell lines as well as PHA-stimulated spleen cells. Culture fluid from 4 of 5 macrophage cell lines contained activity. In contrast, culture fluid from 12 T-cell lines, 9 B-cell lines and 1 mast cell line did not contain activity. The activity found in the culture fluid of one of the macrophage cell lines was located in 2 peaks on Sephadex G-200 chromatography. The position of the peaks was indistinguishable from two peaks found by G-200 chromatography of the culture fluid from a PHA-stimulated spleen cell preparation.

C. Basophils. The mechanism of preferential localization of basophils in certain types of delayed cutaneous hypersensitivity lesions remains unknown. It was previously reported that culture fluids from PHA-stimulated human leukocytes increased the chemotactic response of basophils to complement C5a. This was of particular interest since among the various types of leukocytes the enhancement was restricted to basophils. A molecule of 35,000 molecular weight was isolated from purified PHA which increases basophil chemotaxis response to C5a or leukocyte-derived chemotactic factor. Antibody induced in rabbits by injection of PHA abolished both mitogenic and chemotaxis-enhancing effects of PHA. It also eliminated the basophil chemotaxis augmentation effects of culture fluids from PHA-stimulated leukocytes, showing that the augmentation effect of culture fluids is due to a molecule in the PHA preparation and not to a product of stimulated leukocytes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08525-02 LIB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Primary Autochthonous Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. J. Rapp Chief, Laboratory of Immunobiology LIB NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Immunobiology, Immunology Program

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animals with experimentally induced or naturally occurring primary autochthonous cancer are being studied as guides to the immunotherapy of human cancer. It is hoped that these guides will provide information more relevant to the treatment of human cancer than that derived from studies with transplanted tumors. Cattle with ocular squamous cell carcinoma ("cancer eye") have been treated successfully by the injection of BCG cell wall vaccine directly into the tumor.

Project Description

Objectives: The primary objective of this project is to test a variety of immunologic cancer therapies in several species with primary cancer in the hope of providing guides to the immunotherapy of human cancer. The therapeutic methods tested will be those that have been successful in animal models consisting of syngeneic tumor transplants.

Methods Employed: Hereford cattle were examined at feed lots for evidence of "cancer eye". Suspected animals were purchased and transported to Colorado State University. Diagnosis was established by microscopic examination of stained smears from lesions. Animals with papillomas were not entered into the treatment protocol. Cattle with histologically confirmed carcinoma were divided into three groups. Twenty-four animals in group I received a single treatment consisting of multiple injections of BCG cell wall vaccine into the tumor. Nine animals in group II received the ingredients of the vaccine deliberately compounded improperly. Nine animals in group III received no treatment.

Major Findings: Eight of nine untreated animals have been killed because of advanced malignant disease. Survival time for these animals ranged from three to twenty-four months from the time of diagnosis to death. One of the nine untreated animals is still alive and may constitute an authentic spontaneous cure. All nine animals that received sham vaccine have died; their survival time ranged from three months to twenty-seven months. Sixteen of twenty-three animals treated with BCG cell wall vaccine have died; their survival time ranged from five to thirty-two months. Seven treated animals are alive and the time from their treatment to the present ranges from eighteen to twenty seven months. Three of the treated animals that died remained free of primary disease but acquired secondary lesions. In a separate study local surgery is being compared to intralesional BCG cell wall vaccine in the treatment of bovine ocular squamous cell carcinoma. At present the number of animals alive in the two groups is about the same.

Significance to Biomedical Research and the Program of the Institute: Clinical cancer immunotherapy has been proceeding with minimal guidance from the experimental laboratory. The lack of fundamental information for the design of clinical protocols has necessitated an empirical rather than a rational approach to the immunotherapy of human cancer. Among current necessary practices in cancer immunotherapy are 1) The use of immunotherapy in advanced malignancy after other forms of treatment, 2) Multiple treatments with immunologically active agents that are safe if given once but potentially lethal when given more than once, 3) Administration of agents by routes that are not curative in animals models, 4) Modification of clinical trial protocols while a study is in progress and 5) The use of historical controls in clinical trials rather than randomization. Practices of these kinds have generated information that may discourage appropriate application of immunologic principles to the control of human cancer. One possible factor contributing to this situation is the lack of suitable animal studies to provide the information necessary to design rational clinical protocols. Bovine ocular squamous cell carcinoma provides a potential guide lacking the undesirable aspects of currently available experimental models and perhaps the means to establish useful approaches to the immunotherapy of human cancer. Among the similarities to human cancer and advantages of the bovine model are: 1) The size of the animal makes it possible to take large tissue samples and quantities of blood for study without adverse effects on the animal. 2) The animals are long lived thus permitting an adequate follow up. 3) Surgical techniques and drug dosages are similar to those used in humans. 4) Potentially metastatic, primary autochtonous carcinomas that develop and grow slowly are the major cause of humans cancer deaths. 5) Exfoliative cytology can be done without surgery. 6) It may be possible to delineate the factors that contribute to the failure of the immune system to control the development of naturally occurring cancer. 7) Thousands of animals with "cancer eye" are available in the United States as well as other countries where large numbers of cattle are raised. 8) Studies with "cancer eye" are reasonable because the expense during carcinogenesis is borne by the raiser and not the investigator and the cost of housing each animal compares favorably with smaller, laboratory housed animals.

Proposed Course of Project: Mycobacterial preparations with chemically defined ingredients are now available; they have been found to be effective in the treatment of transplantable tumor. These defined agents will be used in further trials of the immunotherapy of bovine "cancer eye". Studies have recently been started in the immunotherapy of naturally occurring primary breast cancer in dogs, and in inbred mice, primary lymphoma in inbred mice, primary melanoma in horses, chemically induced liver cancer in monkeys and chemically induced primary colon cancer in rats. Radical surgery will be compared to immunotherapy in the treatment of bovine "cancer eye".

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08526-02 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunologic Approaches to the Treatment of Animals with Stage I Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	B. Zbar	Senior Investigator	LIB NCI
OTHER:	J. Hunter	Expert	LIB NCI
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TOTAL MANYEARS: 2.5	PROFESSIONAL: 2.0	OTHER: 0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by methods involving immunostimulation. The areas of current interest are 1) the determination of conditions required for immunologic eradication of lymph node metastases and 2) the study of the cellular and molecular basis of eradication of tumor cells within lymph nodes.

Project Description

Objectives: The primary objective of this project is to define the conditions and methods best suited to the prevention and treatment of cancer by procedures involving immunostimulation. This objective is based on the finding that tumors contain unique antigens capable of eliciting the formation of specific humoral antibodies and of causing the appearance of cells which react specifically with the tumor antigens.

Methods Employed: Transplantable ascites tumors were derived from primary hepatomas induced in strain-2 male guinea pigs by feeding of diethylnitrosamine. These ascites tumors are maintained by periodic intraperitoneal transplantation. Most of the recent studies have been carried out using the hepatoma line-10 which when injected into the skin produces a progressively growing papule and local metastases within a few days.

Animal model of minimal residual malignant disease. Guinea pigs with established intradermal tumors and microscopic lymph node metastases were treated by excision of dermal tumors. Animals that did not receive additional treatment died from progressive growth of lymph node metastases. Bacterial immunostimulants with or without added tumor antigens were injected after local excision in an attempt to prevent growth of microscopic lymph node metastases.

Intralesional immunotherapy of cutaneous tumors. Guinea pigs with established dermal tumors and microscopic lymph node metastases were treated by intralesional injection of varying doses of living BCG or BCG CW.

Intralesional immunotherapy of oral tumors. Line-10 tumor cells were injected into the buccal pads of inbred strain-2 guinea pigs. Animals were treated by local excision or by intralesional injection of living BCG or BCG CW 6 days after injection of tumor cells.

Time required for eradication of lymph node metastases. At various intervals after intralesional injection of BCG CW, axillary lymph nodes were removed, disaggregated and quantitatively transferred to the peritoneal cavity of normal weanling strain-2 guinea pigs.

Ability of animals treated by intralesional injection to reject distant metastases. On the day of intralesional treatment, guinea pigs were challenged intradermally, intravenously or intraperitoneally with varying doses of tumor cells. The ability of intralesional treatment to control the growth of contralateral lymph node metastases was also evaluated. Guinea pigs received intradermal injections of line-10 into the left flank on day 0. At various intervals after injection of tumor into the left flank, tumor cells were injected into the right flank. Tumors on the left flank were treated by intralesional injection on day 7; tumors on the right flank were locally excised on day 7.

Major Findings: Development of an animal model for immunotherapy of minimal residual malignant disease. Bacterial immunostimulants injected adjacent to the site of excision had a weak but definite antitumor effect. Microscopic lymph node metastases were eradicated in 15-30% of treated animals. Bacterial immunostimulants injected contralateral to the sites of excision had no significant antitumor effect. Vaccines containing bacterial immunostimulants and tumor antigen were significantly more effective in controlling growth of metastases than were vaccines containing bacterial immunostimulants alone. Microscopic lymph node metastases were eradicated in about 90% of animals treated by ipsilateral injection of immunostimulants and tumor antigens and in 50-70% of animals treated by contralateral injection of immunostimulants and tumor antigens. Results obtained with nonliving bacterial immunostimulants were more consistent than results obtained with living BCG.

Safety and efficacy of living BCG or BCG cell walls in the treatment of a guinea pig hepatoma. The lowest dose of living BCG used produced a significant cure rate and no grossly evident toxicity. An intermediate and the highest dose of living BCG used cured some animals but others lost weight and a significant number died as a result of the treatment. Histologic examination of animals with significant weight loss showed fatty degeneration of the liver, granulomatous hepatites and histiocytic infiltration of the spleen. None of the doses of BCG CW used was toxic and they were at least as effective as living BCG in intralesional treatment. In some experiments in which treatment was delayed, it was found that the extent of disease required to render treatment ineffective was about the same for living BCG and for BCG CW.

Regression of established oral tumors after intralesional injection of living BCG or BCG cell walls. Oral tumors with associated cervical lymph node metastases developed after injection of tumor cells into buccal pads of inbred guinea pigs. Intralesional injection of living BCG or BCG cell walls caused regression of established tumors, prevented the development of cervical lymph node metastases and led to the development of host resistance to the growth of a subsequent tumor transplant.

Ability of animals treated by intralesional injection to reject distant metastases depends upon the anatomic location of metastases. Guinea pigs treated by intralesional injection of BCG CW were able to prevent the growth of artificial metastases present in skin or lung but failed to prevent the growth of artificial metastases present in the peritoneal cavity or contralateral axillary lymph nodes.

Time required for immunologic eradication of metastases. Viable tumor cells were found in association with excised lymph nodes of all animals on the day of intralesional treatment. Viable tumor cells were present in axillary lymph nodes of 70% of animals 7 days after treatment and were present in 10% of animals 20 days after treatment.

Significance to Biomedical Research and the Program of the Institute: Clinical attempts to retard growth of minimal residual malignant disease by immunotherapy have been unsuccessful. The experiments described above suggest some possible reasons for this failure and fruitful avenues for future clinical investigation. Clinical evaluation of efficacy of intralesional immunotherapy of bacterial immunostimulants has been complicated by significant toxicity from living BCG. Data presented above indicate that the nonliving immunostimulant, BCG CW, possesses antitumor activity equivalent to living BCG and lacks systemic toxicity. Use of this nonliving immunostimulant in clinical trials may avoid the toxicity associated with high doses of living BCG and provide a clearer picture of the contribution of mycobacterial adjuvant to resistance to tumor growth. The demonstration that after intralesional injection of BCG CW oral tumors regress and the growth of cervical lymph node metastases is prevented provides support for a clinical trial of this therapy in patients with head and neck cancer.

Proposed Course of Project: Attempts will be made to improve the potency of vaccines effective in controlling minimal residual malignant disease. Factors that will be investigated include: dose of nonliving bacterial immunostimulants, oil, and tumor antigen. Included in these tests, will be determinations of proportions of bacterial immunostimulants to tumor antigens optimal for eradication of metastases. The efficacy of X-irradiated tumor cells in these vaccines will be evaluated. Other tumors will be substituted for Line-10 to formally demonstrate that the effects observed require antigenic identity of tumor used for immunization and tumor in metastases. The limits of effective therapy will be defined. The ability of vaccines to control growth of microscopic metastases in proximal axillary lymph nodes, brain, bone and lung will be evaluated.

Publications

Zbar, B., Ribi, E., and Rapp, H.J.: Immunotherapy of a guinea pig hepatoma with living BCG: A frozen liquid and a lyophilized preparation are equally active. Cancer 40: 2930-2932, 1977.

Zbar, B., Hunter, J., Rapp, H.J., and Canti, G.: Safety and efficacy of living BCG or BCG cell walls (CW) in the treatment of a guinea pig hepatoma. Cancer., in press.

Zbar, B., Canti, G., Rapp, H.J., Bier, J. and Borsos, T.: Regression of established oral tumors after intralesional injection of living BCG or BCG cell walls. Cancer., in press.

Zbar, B., Hunter, J. T., Rapp, H.J. and Canti, G.F.: Immunotherapy of bilateral lymph node metastases by intralesional or paralesional injection of M. bovis (BCG). J. Natl. Cancer Inst. 60: 1163-1168, 1978.

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PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunologic Approaches to Treatment of Animals with Stage II Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by treatments involving immunostimulation. The areas of current interest are: 1) the development of immunologic methods effective in the treatment of animals with stage II malignant disease and 2) evaluation of tumor immunity in tumor bearing animals and in animals rendered free of apparent malignant disease by surgery.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The primary objective of this project is to determine the role of systemic immunity in the antitumor effects of immunoadjuvant therapy of stage II malignant disease or systemic metastases.

Methods Employed: A transplantable syngeneic hepatocellular carcinoma, designated line-10, in transplant generations 8-18 was used. This diethylnitrosamine-induced tumor had been converted to an ascites form and is passed ip in weanling strain-2 male guinea pigs. Inoculation of 10^6 line-10 tumor cells ID leads to progressive tumor growth and metastasis to the regional axillary lymph node by 7 days after tumor implantation.

Intradermal tumor challenge is carried out by injecting 10^6-10^4 cells ID into the skin of the contralateral flank. Challenge sites were examined weekly for 3 consecutive weeks after injection for evidence of tumor growth.

Surgery is carried out utilizing ether and pentobarbital anesthesia. The dermal tumor is excised with a skin margin and the draining lymph node chains are dissected out.

Intralesional BCG therapy is carried out by injecting the tumor with 0.4 ml of an oil saline emulsion containing 850 μg of BCG cell wall.

A model for treatment of clinically detectable lymph node metastases by radical lymph node dissection and BCG therapy was used in which one million line-10 cells were injected intradermally into strain-2 guinea pigs. When palpable lymph node metastases were detected, BCG was injected into the tumor and 7 days later the tumor was excised and regional lymph node dissection performed. Palpable metastases were first detectable when the nodes were 6-7 mm in diameter, approximately 21 days after tumor implantation.

Tumor "metastases" in the lungs were established by the injection of 10^4 viable line-10 tumor cells into the penile vein of strain-2 guinea pigs on the day prior to therapy.

Major Findings: Animal model for stage II malignant disease. Past results of this project indicated that intralesional injection of BCG prior to radical surgery failed to augment the efficacy of surgery when treatment was instituted at the time of detection of palpable lymph node metastases. Current results indicate that tumor deposits implanted in the lungs by intravenous injection of tumor cells 24 hours prior to treatment by intralesional BCG combined with surgery to remove the dermal tumor and axillary lymph nodes significantly prolonged the survival of animals treated when palpable lymph node metastases were present. Neither therapy alone was effective under the above conditions. Data from experiments on stage I disease described elsewhere in this report indicate that tumor in lymph nodes may be relatively resistant to systemic immunity.

Tumor immunity in animals cured of tumor by radical surgery. Radical surgery led to detectable resistance to tumor challenge only in certain situations. The rate of development of resistance to tumor challenge was dependent upon the amount of tumor initially implanted. The development of resistance to tumor challenge required the presence of the dermal tumor transplant until the time of radical surgery. Radical surgery performed when draining lymph node metastasis was palpable rendered animals resistant to a contralateral challenge of the line-10 tumor. Resistance was usually not seen when surgery was performed before the development of palpable metastases. Neither concomitant immunity nor resistance to challenge in animals with recurrent tumor was seen. Resistance to intradermal challenge of 10^4 or 10^5 tumor cells was detectable even when the challenge was given immediately after curative surgery.

Significance to Biomedical Research and the Program of the Institute. Human therapy with intralesional BCG has been most successful in treatment of stage I dermal tumors and it has been reported to prevent recurrence after surgery of stage I squamous cell carcinoma of the lung. Therapy of stage II or more advanced disease has not been strikingly effective.

The relationship between intralesional BCG injection, the development of systemic tumor immunity and the effects on local and systemic metastatic disease may elucidate the difficulties.

Proposed Course of Project: To continue to conduct experiments on treatment of stage II or more advanced cancer by immunotherapy and surgery combination in our guinea pig model and in spontaneously metastasizing syngeneic mouse tumor models.

Publications

Rapp, H. J., and Hunter, J. T.: Animals, test-tubes and cancer: In vivo veritas, in vitro mendacium. In, Proceedings of the Vatican Study Week on "The role of non-specific immunity in the prevention and treatment of cancer". Vatican City, Rome. October 17-21, 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08528-02 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanism of Delayed Hypersensitivity and Tumor Graft Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB NCI
	B. Zbar	Senior Investigator	LIB NCI
OTHER:	M. P. Ashley	Visiting Fellow	LIB NCI
	G. Canti	Visiting Fellow	LIB NCI
	M. S. Meltzer	Surgeon	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunobiology, Immunology Program

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to elucidate the mechanism of the immunologic rejection of tumor grafts and to determine the mechanism of tumor suppression following immunostimulation with BCG. Current areas of investigation are: comparison of ability of endotoxin responsive (C3H/HeN) and endotoxin unresponsive (C3H/HeJ) mice to suppress tumor growth at sites of BCG infection and to reject syngeneic tumor grafts; determination of optimal conditions for immunization against transplantable syngeneic murine tumors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project are: to define the cellular and molecular basis of BCG mediated suppression of tumor growth and of rejection of syngeneic tumor grafts, and to determine conditions required for optimal immunization against syngeneic tumors.

Methods Employed: Animals. Male C3H/HeN mice were obtained from the Laboratory Aids Branch, Division of Research Services, NIH; Male C3H/HeJ mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Tumors. Four tumor lines, designated 1023, 1037, 1038 and 1063 were induced in the subcutaneous tissue of C3H/HeNIcr male mice with pellets of 1% 3-methylcholanthrene in paraffin. Tumor cell suspensions were prepared by enzymatic digestion of minced tumors.

BCG mediated tumor suppression and regression. In suppression tests tumor cells were mixed with varying doses of living BCG in vitro and this mixture was injected into the skin of syngeneic mice. Tumor growth was measured at weekly intervals. At the end of the observation period, the number of BCG required to inhibit tumor growth in 50% of mice (Inhibiting dose$_{50}$, ID$_{50}$) was calculated by the method of Reed and Muench. In regression tests, syngeneic tumor cells were injected intradermally. When established tumors were present, bacterial immunostimulants were injected directly into the tumors. Different doses of tumor cells and adjuvants were mixed in vitro and these mixtures were injected into the skin of syngeneic mice. Vaccination sites were excised 8 days after injection; excised skin sites were placed in fixative and saved for future histologic examination. Animals were challenged intradermally 16 days after vaccination with graded doses of tumor cells.

Major Findings: Comparison of ability of endotoxin responsive (C3H/HeN) and endotoxin unresponsive (C3H/HeJ) mice to inhibit tumor growth. C3H/HeJ mice required about 20 times more BCG than C3H/HeN mice to inhibit tumor growth. C3HeB/FeJ mice, another endotoxin responsive strain required about the same amount of BCG to inhibit tumor growth as C3H/HeN mice. Established tumors failed to regress in C3H/HeJ mice after intralesional injection of 10^7 living BCG. In contrast, established tumors regressed in C3H/HeN mice after intralesional injection of 10^7 living BCG.

C3H/HeN and C3H/HeJ mice free of tumor as a consequence of mycobacterial infection were able to reject 1×10^6 or 5×10^6 tumor cells given as an intradermal challenge inoculum. These challenge doses were approximately 100 to 500 x the LD$_{50}$ for this tumor.

Conditions required for optimal immunization against syngeneic tumors. Sixteen combinations of bacterial immunostimulants (living BCG) and tumor cells were evaluated for ability to evoke host resistance to tumor challenge. Three of the sixteen combinations were effective in inducing host resistance to tumor growth. The proportions of BCG and tumor cells optimal for producing tumor immunity were: 10^6 tumor cells admixed with 2 X 10^7 BCG, and 10^5 tumor cells admixed with 2 x 10^6 or 2 x 10^7 BCG. Immunization by intradermal growth and excision enabled mice to resist approximately 5 $LD_{50's}$. Immunization with BCG and tumor cells enabled mice to resist approximately 60 $LD_{50's}$.

Significance to Biomedical Research and the Program of the Institute: Determination of optimal conditions for immunization against weakly immunogenic murine tumors may lead to principles for formulation of effective vaccines. This information would be valuable in designing human trials of active immunotherapy.

Improved understanding of the host factors controlling the success of intralesional immunotherapy may lead to more effective application of this modality of therapy in patients with cancer.

Proposed Course of Project: (1) To study other histocompatible mouse strain pairs that differ in endotoxin responsiveness for ability to inhibit tumor growth at sites of BCG infection. (2) To determine whether the gene(s) controlling host ability to suppress tumor growth at sites of BCG infection is linked to the Lps gene. (3) To determine whether there is a difference in the ability of C3H/HeJ and C3H/HeN mice to suppress tumor growth at sites of injection of other bacterial immunostimulants (BCG cell walls on oil droplets, Corynebacterium parvum). (4) To test whether C3H/HeJ and C3H/HeN mice differ in response to infection with Moloney sarcoma virus. (5) To determine optimal conditions for immunization against spontaneous murine tumors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08529-02 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunotherapy with Defined Bacterial Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB NCI
	E. Yarkoni	Visiting Fellow	LIB NCI
OTHER:	L. Ruco	Visiting Fellow	LIB NCI
	M. Meltzer	Surgeon	LIB NCI

COOPERATING UNITS (if any)

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National Jewish Hospital and Research Center, Denver, Colorado

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Cellular Immunity Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Components of mycobacteria injected in oil/water emulsions into syngeneic tumors growing in the skin of guinea pigs or mice caused the tumors to regress. Animals cured of their tumors were resistant to rechallenge with the same tumor.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The main objective of this project is to isolate and define mycobacterial components with maximal antitumor effects and minimal toxicity.

Methods Employed: Malignant hepatomas were induced in strain-2 guinea pigs by the feeding of diethylnitrosamine. Ascites variants of the tumors were maintained by serial intraperitoneal passage.

Fibrosarcomas arose in the subcutaneous tissues of C3H/HeNIcr male mice after implantation of paraffin pellets containing 1% 3-methylcholanthrene. The tumors were maintained by serial trocar passage in syngeneic immunodepressed mice.

Mineral oil/water emulsions of mycobacterial vaccines were prepared by ultrasonification or grinding. The following mycobacterial components were investigated: BCG cell walls, methanol extraction residue (MER) of BCG cells, trehalose-6,6'-dimycolate (TDM), sulfolipid I and sulfolipid III.

Emulsified mycobacterial vaccines were infiltrated into the growing tumors. The tumors were subsequently observed for progression or regression.

Mycobacterial vaccines were injected intravenously into normal mice and their weight and deaths were recorded. Histopathology studies were conducted in mice after iv or intralesional inoculation of TDM or C76 (a synthetic analog of TDM).

Major findings: (1) Injection of emulsified BCG cell walls or MER into transplants of an established, syngeneic murine fibrosarcoma induced complete regression of tumor in a number of mice. The number of animals in which tumors regressed completely depended on the amount of oil in the emulsion. (2) BCG cell wall skeletons (SK) derived from BCG cell walls (CW) by treatment with proteolytic enzymes and organic solvents were tested for their potency to cause regression of a transplanted guinea pig hepatoma. On a weight basis, SK were as effective as CW in causing tumor regression and they as well as purified protein derivative of mycobacteria provoked delayed cutaneous hypersensitivity (DCH) reactions in animals immunized with CW or with SK. On a weight basis, CW were more active than SK in eliciting DCH in sensitized guinea pigs whether the animals were immunized with CW or with SK. In unimmunized animals the inflammatory response to intradermally administered CW was greater than that evoked by SK. CW and SK provoked DCH reactions of similar strength in animals immunized with living BCG. This study provided no compelling reasons for using SK instead of CW in clinical trials of cancer treatment by mycobacterial vaccines. (3) Emulsions of mycobacterial sulfolipids I or III in admixture with endotoxin were able to cause regression of a transplanted guinea pig hepatoma. Emulsions of sulfolipid I or II alone were neither granulomagenic nor toxic after iv administration into mice, and were ineffective in causing regression of transplants of an established murine fibrosarcoma. (4) Injection of emulsified 6,6'-di-O-2-tetradecyl-3-hydroxyoctadecanoyl- α,α -

trehalose, designated C76, a synthetic analog of the mycobacterial glycolipid trehalose-6,6'-dimycolate (TDM), into transplants of an established syngeneic murine fibrosarcoma induced complete regression of tumor in a number of animals. The numbers of animals in which tumors regressed completely depended on the amount of oil in the emulsion. Emulsions of C76 were less toxic and less granulomagenic in mice when compared with TDM. Emulsified preparations of C76 in admixture with endotoxin were able to cause regression of a transplanted guinea pig hepatoma. Neither emulsified ET alone nor emulsified C76 alone were able to cause regression of the guinea pig hepatoma (5) The antitumor activity of emulsified TDM, TDM + endotoxin, and emulsified turpentine was compared. Preparations were injected into established murine fibrosarcomas. The tumors regressed completely in the skin site after the treatment with these agents. The histological changes in the injected sites induced by these agents were similar: local necrosis and marked infiltration with neutrophils, which was followed by infiltration of mononuclear cells and reparative fibrous tissue. In the regional lymph nodes depletion of the paracortical area and histocytosis were evident after the treatment with TDM or turpentine. Treatment with endotoxin induced a marked increase in the size of the regional lymph nodes and lymphocytic proliferation occurred in the B cell dependent areas. No granulomas were found in the injected site or in the draining lymph nodes after the treatment with TDM. Results of this study do not lend support to the hypothesis that granulomagenic activity is required for antitumor action of TDM. (6) Intralesional injection of emulsified croton oil into transplants of an established, syngeneic murine fibrosarcoma or guinea pig hepatoma caused complete regression of the primary tumors. In guinea pigs the development of the microscopic lymph nodes metastases were delayed but not eradicated and the guinea pigs died of progressive tumor growth. TPA (12-O-tetradecanoyl-phorbol-13-acetate) is one of the active principles of croton oil. Intralesional injection of mineral oil-in-water emulsions of TPA caused regression of murine tumors. In contrast, aqueous suspension of TPA failed to eradicate the tumors.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy with BCG cell walls and trehalose-6,6'-dimycolate with or without endotoxin has proven to be effective treatment when the tumor mass is small and microscopic metastases are present. There are clinical situations in which therapy with mycobacterial vaccines may be a practical and desirable approach. Killed BCG cell vaccines with or without trehalose-6,6'-dimycolate have been tried successfully in preliminary studies of the treatment of human skin cancer.

Proposed Course of Project: Experiments designed to determine the requirements for successful immunotherapy will be continued. Emphasis will be placed on the isolation and definition of additional components of mycobacteria which cause regression of dermal tumors and palpable lymph node metastases. Synthetic analogs of mycobacterial components will be investigated for their antitumor activities. Attempts will be made to replace the mineral oil with digestible oil(s) in the mycobacterial vaccines.

Publications

Yarkoni, E., and Rapp, H.J.: Granuloma formation in lungs of mice after intravenous administration of emulsified trehalose-6,6'-dimycolate (cord factor): Reaction intensity depends on the size distribution of oil droplets. Infect. Immunity 18: 552-554, 1977.

Yarkoni, E., and Rapp, H.J.: Toxicity of emulsified trehalose-6,6'-dimycolate (cord factor) in mice depends on the size distribution of the mineral oil droplets. Infect. Immunity, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08550-04 LIB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Modification of Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. I. Schlager	Staff Fellow	LIB NCI
OTHER:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	M. D. P. Boyle	Expert	LIB NCI
	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Immunobiology, Immunology Program

SECTION

Humoral Immunity Section

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TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.1	OTHER: 0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pretreatment of tumor cells with chemotherapeutic agents, metabolic inhibitors, enzymes or hormones modifies the susceptibility of the cells to killing by antibody and complement. The purpose of this investigation is to determine the attributes of the cell which are modified following treatment with the agents.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To study the mechanisms whereby enzymes, metabolic inhibitors, and anti-lipidemic agents increase the sensitivity of tumor cells to immune attack; 2) to study mechanisms whereby hormones decrease sensitivity of cells to immune attack; 3) to determine the physical and chemical processes and cell products which may be modified following such treatment; 4) to determine the processes which may be modified following treatment with antibody and complement (C).

Methods Employed: Antibody is quantitated by the C fixation and transfer test and immune cytolysis. Sensitivity of cells to attack is measured by uptake of trypan blue and/or release of ^{125}IUdR. Incorporation of radiolabelled precursors of DNA, RNA, protein, complex carbohydrate, glycoproteins, glycolipids and lipids are being used to measure general metabolic properties of the cells. Thin layer chromatography is being utilized to analyze and identify specific lipid moieties associated with the cells. Sucrose density gradient ultracentrifugation is used to prepare plasma membrane and intracellular membrane fractions of tumor cells.

Major Findings: The ability of line-1 and line-10 tumor cells to resist humoral immune attack correlated with their ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate. Cells treated for short periods or with low doses of metabolic inhibitors or chemotherapeutic agents are not rendered susceptible to killing and are not inhibited in their lipid synthesis; these cells are inhibited in their synthesis of DNA, RNA, protein, and complex carbohydrate. Prolonged incubation of the cells with high concentrations of drugs renders the cells sensitive to killing and inhibits their ability for complex lipid synthesis. Both of these effects reverse within 4 hr of reculture without drug; the cells do not regain their ability to synthesize DNA, RNA, protein, or complex carbohydrate.

Tumor cells irradiated with 6,000 or 9,000 R are rendered susceptible to antibody-C mediated killing 6 hr post-irradiation and remain sensitive 16 hr post-irradiation. Cells heated 30 min at 43, 44 or 45°C do not become susceptible. The sensitivity or resistance of the tumor cells following either physical treatment correlated with their inability or ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate.

Line-1 tumor cells incubated for 1 hr with a 5×10^{-4} M concentration of the antilipidemic drug, Atromid-S, were rendered sensitive to killing by antibody plus GPC. These cells were inhibited in their lipid synthesis, but not in their ability to synthesize DNA, RNA, or protein.

Thin layer chromatography of $CHCl_3:CH_3OH$ lipid extracts obtained from drug-treated cells indicated that the drug-induced increase in susceptibility to antibody-C mediated killing correlated with the inhibition of incorporation of acetate, glycerol, and fatty acids into triglycerides and/or specific phospholipids (cardiolipin and phosphatidyl choline) in the cells. A generalized inhibition of the synthesis of other lipid moieties by drug treatment was not observed. Line-1 tumor cells treated with effective drugs that were rendered sensitive to killing by anti-Forssman antibody plus GPC, but not specific antitumor antibody plus GPC, were inhibited in their incorporation of saturated (palmitic and stearic) fatty acids, but not in their incorporation of an unsaturated (linoleic) fatty acid. Cells rendered sensitive to drug treatment to killing by either antibody plus GPC were inhibited in their incorporation of both saturated and unsaturated fatty acids.

Glucocorticoid and polypeptide hormones render line-1 and line-10 guinea pig hepatoma cells more resistant to killing by antibody and C. The effect is dose and temperature dependent and reverses even in the continued presence of cell-bound hormone. Hormone-treated reversed cells can be rendered resistant to killing after incubation with a different, but not with the same, hormone. In addition, hormone pretreatment of line-10 cells reduces their sensitivity to killing following treatment with proteolytic enzymes. The metabolic requirements for hormones to be effective in rendering tumor cells resistant to antibody-C mediated killing are that cells have at least RNA and protein synthesis intact. There is no absolute requirement for DNA synthesis.

The hormone-induced increase in resistance of the cells to antibody-C mediated killing was accompanied by an enhancement in complex lipid synthesis by the cells as measured by the incorporation of acetate, glycerol and fatty acids into cellular lipids. The enhancement in lipid synthesis following hormone treatment affected specific phospholipids (phosphatidyl choline and phosphatidyl serine) and, occasionally, triglycerides. No generalized enhancement of the synthesis of other cellular lipids was noted as a result of hormone treatment.

Antibody-sensitized line-1 and line-10 tumor cells treated with GPC (TAC) incorporated increased amounts of fatty acids into complex cellular lipids and released increased amounts of fatty acids within 5 to 10 min after the addition of GPC as compared to control cells. This effect was dependent on the concentration of GPC used; however, under conditions where the cells were not killed, the enhanced synthesis and release of lipids were not dependent on the antibody concentration used to sensitize the cells. Treatment of the cells with antibody alone, GPC alone, or antibody plus heat-inactivated GPC did not result in enhanced synthesis or release of lipids. No enhancement in DNA, RNA, or protein synthesis in TAC was noted. Line-1 cells, which can be killed by GPC when sensitized with excess anti-Forssman IgM antibody, demonstrated enhanced lipid synthesis within 1 to 3 min after the addition of GPC to the antibody-sensitized cells, before measurable killing of the cells had occurred. This effect persisted in the surviving cells when tested 5 and 10 min after the formation of TAC. Addition of GPC deficient in C4 to antibody-sensitized cells did not result in enhanced lipid synthesis or release.

Thin layer chromatography of lipids from antibody-GPC treated cells indicated that the cells uniformly synthesized and released increased amounts of the phospholipid, cardiolipin. Modifications in the synthesis and/or release of triglycerides, phosphatidyl ethanolamine, phosphatidyl choline, cholesteryl esters, or phosphatidyl serine were also observed in line-1 or line-10 cells prelabelled with a particular precursor of lipid synthesis. Treatment of antibody-sensitized tumor cells with HuC did not cause an increase in the incorporation of acetate, glycerol, or fatty acids into lipids, but caused a shift in lipid synthesis from cardiolipin to phosphatidyl choline. The cells treated with antibody plus HuC released increased amounts of cholesteryl esters and triglycerides.

Significance to Biomedical Research and the Program of the Institute: Modification of tumor cells by metabolic inhibitors, hormones, chemotherapeutic agents and antilipidemic agents furnishes a new tool to study the interaction of tumor cells with the immune defense mechanisms of the host. The study of the response by tumor cells to humoral immune attack through modifications in various cellular metabolic pathways provides information regarding the mechanism of defense or cytomembrane repair processes in these cells. Modification of these processes may lead to cells that are more vulnerable to immune attack mechanisms.

Proposed Course of Project: Radioisotope incorporation studies will be continued as probes to determine the cellular functions which are modified by treatment with drugs or hormones. The chemical attributes of normal cells, tumor cells and treated tumor cells will be studied. Work will continue on the effect of enzymes, hormones and inhibitors of macromolecular synthesis on the metabolic function of the tumor cells. Analysis of membrane-associated and intracellular macromolecules in these cells will be pursued. Further chemical analysis of the lipid and fatty acid composition of cells that are susceptible or resistant to antibody-C mediated killing will be made.

Publications

Schlager, S. I., Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Effect of inhibiting DNA, RNA, and protein synthesis of tumor cells on their susceptibility to killing by antibody and complement. Cancer Res. 37: 1432-1437, 1977.

Schlager, S. I. and Ohanian, S. H.: Correlation between lipid synthesis in tumor cells and their sensitivity to humoral immune attack. Science 197: 773-776, 1977.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Correlation between the ability of tumor cells to incorporate specific fatty acids and their sensitivity to killing by a specific antibody plus GPC. J. Natl. Cancer Inst., in press.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Correlation between the ability of tumor cells to resist humoral immune attack and their ability to synthesize lipid. J. Immunol. 120: 463-471, 1978.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Identification of lipids associated with the ability of tumor cells to resist humoral immune attack. J. Immunol. 120: 472-480, 1978.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Identification of lipids synthesized and released by tumor cells under attack by antibody and complement. J. Immunol. 120: 1644-1650, 1978.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Metabolic requirements for hormone-induced resistance to antibody-complement killing of tumor cells. J. Immunol. 119: 789-794, 1977.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Stimulation of the synthesis and release of lipids in tumor cells under attack by antibody and complement. J. Immunol. 120: 895-901, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08551-03 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Induction of Tumor Immunity by Chemotherapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI
OTHER:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	S. I. Schlager	Staff Fellow	LIB NCI
	E. Yarkoni	Visiting Fellow	LIB NCI
	B. Zbar	Senior Investigator	LIB NCI
	J. T. Hunter	Expert	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Selected chemotherapeutic agents injected into tumors growing in vivo cause the tumor to regress. Animals cured of their tumors are resistant to rechallenge with the same tumor. The purpose of this work is to analyze the mechanisms of intralesional chemotherapy and include histological techniques, and methods for analyzing humoral and cellular mediated immune mechanisms.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To cure tumors and induce tumor immunity by direct treatment of established tumors with chemotherapeutic agents; 2) to study the development of cellular and humoral immunity in cured animals.

Methods Employed: Inhibition of tumor growth in animals cured of tumors by intratumoral chemotherapy is used to measure the development of tumor immunity. Intradermal injections of the ascites form of line-10 tumor cells is used to determine malignancy. Immunological techniques employed to measure humoral and cellular immunity include C fixation, antibody-C mediated killing and cell mediated cytotoxicity.

Major Findings: Due to loss of strain-2 guinea pig colony, work has been temporarily halted.

Significance to Biomedical Research and the Program of the Institute: These studies in a syngeneic tumor animal model system are designed for analyzing the mechanism of intralesional chemotherapy. The development of this system should make it possible to better utilize intratumoral therapy of human cancer.

Proposed Course of Project: Further studies on the effects of intratumoral chemotherapy into tumors growing for longer than 7 days. Histology of the tumor sites at various times after injection of chemotherapeutic agents will be studied. Studies will be performed to determine the effect of multiple small injections of drugs into tumors growing for longer than 7 days. Techniques of humoral and cellular immunity to the tumor will be employed to more critically define the immune status of the treated animals.

Publications

Ohanian, S. H., Yarkoni, E., Hunter, J. T., Okuda, T., Schlager, S. I., Rapp, H. J. and Borsos, T.: Local chemotherapy of a metastasizing guinea pig tumor. In Day, S. B. et al. (Eds.): Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy. New York, Raven Press, pp. 457-463, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08552-12 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanism of Complement Fixation and Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. D. P. Boyle	Expert	LIB NCI
	H. J. Rapp	Chief	LIB NCI
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COOPERATING UNITS (if any)

None

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TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.1	OTHER: 0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a long-range project investigating the mechanism of complement fixation and action. In particular the interaction of antibody-antigen complexes with the first component of complement and the result of this interaction on the other components are investigated. The relation between antibody action and complement activation is also explored. Finally, the significance of complement in the humoral immune defense mechanism is studied.

Project Description

Objectives: To develop new methods and to use available complement (C) fixation tests based on the fixation and transfer of the first component of C (C1) and cytotoxic tests for the analysis of antigen-antibody reactions, in particular in the search for cancer specific antigens.

Methods Employed: The model for studying cytotoxic reactions mediated by antibody and C consists of sheep erythrocytes, hemolytic antibody and guinea pig C (GPC). Purification procedures for antibodies and the C components include: preparative (large-scale) gel filtration, ion exchange chromatography and preparative free electrophoresis. Other techniques used include precipitin and immunoelectrophoretic analysis, analytical, zonal and preparative ultracentrifugation and other immuno- and physico-chemical methods. Development and application of quantitative analytical methods which utilize radiolabelled tracer molecules are being emphasized.

Major Findings: The ultimate goal of this work is the development of immunological tools that permit exploration of 1) immunological approaches to cancer diagnosis and 2) possible host immune defense mechanisms against cancer.

Staphylococcal Protein A (PA) and hemolytic IgG formed complexes that behaved functionally like IgM in their ability to lyse sheep erythrocytes (E) in the presence of whole GPC and to fix purified guinea pig $C\bar{1}$. At relatively high ratios of PA to IgG, the resulting complexes tended to behave functionally like IgG but with reduced hemolytic activity and $C\bar{1}$ fixing ability. Furthermore, the binding of $C\bar{1}$ to EIgG was inhibited by PA and the binding of PA to EIgG was inhibited by $C\bar{1}$ indicating that the binding sites for $C\bar{1}$ and PA were located near each other or were identical. Concanavalin A (Con A), a plant lectin that inhibited IgM but not IgG hemolytic activity, inhibited the hemolytic activity of IgG-PA complexes that behaved like IgM but had no effect on complexes that behaved functionally like IgG. Since Con A is known to bind specifically to glucose and mannose residues, our results suggested that the interaction of PA with the Fc region of IgG led to exposure of sugar moieties that may participate in C binding. Based on the results of hemolytic experiments carried out with specific anti-Forssman IgG and complement, it appeared that IgG not directed against the Forssman antigen could participate with specific antibody in the formation of hemolytically active (i.e. C fixing) IgM-like complexes. The ability of PA to increase the amount of ^{125}I-labelled IgG fraction bound to E under the condition of limiting specific antibody supported this finding. Ultracentrifugation carried out in a sucrose gradient and in the presence of either ^{125}I-labelled PA or IgG was used to separate complexes formed between PA and IgG. By use of standards in these experiments and hemolytic data, the molecular formula of the IgM-like complex was found to be $[(IgG)_2PA]_2$. Depending on the relative amounts of PA and IgG, complexes with molecular formulas corresponding to IgG_2-PA and $[IgG]_3PA_2$ were also formed. These complexes may also be hemolytically active. The IgM-like complex, isolated by centrifugation was stable in terms of hemolytic activity for at least 96 hr at 4°C and at 37°C for at least 24 hr.

Based on the specific binding of PA to IgG, ^{125}I PA has been used in combination with purified rabbit IgG bound to a solid support to develop a competitive binding assay capable of detecting human, rabbit and guinea pig IgG at the nanogram level. An optimal set of assay conditions was established and levels of IgG measured in normal human, rabbit and strain-2 guinea pig serum. ^{125}I PA has also been used to detect IgG anti-Forssman antibody bound to E and to line-1 and line-10 tumor cells and as an indirect assay for tumor-associated antigen in the ascitic fluid of tumor-bearing guinea pigs.

Significance to Biomedical Research and the Program of the Institute: Complement fixation is one of the most widely used diagnostic tools. The development and successful application of a very sensitive C fixation test, the C1FT test, opened up new possibilities in determining antibody-antigen reactions on cell surfaces.

Furthermore, cytotoxic reactions due to antibody and C are prime examples of body defense mechanisms. Fundamental research into the nature and mechanism of C fixation and action will contribute greatly to the development of diagnostic tools and to the understanding of the mechanism of immune body defenses.

Proposed Course of Project: This is a long-range project, and little change is expected in the scope of the work during the next few years. The ultimate goals of this project are the development of better diagnostic tools and the elucidation of molecular events associated with the action of C and antibodies. It is hoped that as a result of our program of inquiry into the basic problem of the interaction of antibodies, antigens and components of C, tools will be developed that are of practical significance in the search for cancer antigens.

Publications

Boyle, M. D. P., Langone, J. J. and Borsos, T.: Effect of lectins on the hemolytic activity of complement components. Immunochemistry, in press.

Boyle, M. D. P., Langone, J. J., Ohanian, S. H. and Borsos, T.: Effect of Concanavalin A on the killing of tumor cells by antibody and complement. J. Immunol. 118: 1626-1630, 1977.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Effect of Concanavalin A on the classical complement pathway. J. Immunol. 118: 1622-1625, 1977.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Effect of Concanavalin A on the functional activity of hemolytic antibody. Immunol. Commun. 6: 283-296, 1977.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: ^{125}I Protein A: Applications to the quantitative determination of fluid phase and cell-bound IgG. J. Immunol. Methods 18: 281-293, 1977.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Studies on the interaction between Protein A and immunoglobulin G. I. Effect of Protein A on the functional activity of IgG. J. Immunol., in press.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Studies on the interaction between Protein A and immunoglobulin G. II. Composition of complexes formed between Protein A and IgG. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08553-09 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immune Cytolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. D. P. Boyle	Expert	LIB NCI
OTHER:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI
	S. I. Schlager	Staff Fellow	LIB NCI
	A. P. Gee	Visiting Fellow	LIB NCI
	J. J. Langone	Senior Staff Fellow	LIB NCI
	T. Lo	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

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Laboratory of Immunobiology, Immunology Program

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	2.2	0.2

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the molecular aspects of the killing of nucleated cells by immune attack. In particular the interaction of antibody (class, affinity, etc.) and complement with one another and with the cell surface.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: A study of the molecular aspects of antibody-complement lysis and antigen topography of nucleated cells.

Methods Employed: Antibodies to normal and tumor antigens are detected and quantified by immune cytolysis, colony inhibition, C1 fixation and transfer (C1FT), uptake of ^{125}I labelled antibody, and by binding of ^{125}I labelled Protein A. Immunochemical methods, including Sephadex and DEAE chromatography, electrophoresis, immunodiffusion and ultracentrifugation are employed to isolate antibody, protein and complement (C) components.

A cytotoxic assay to quantify antibody-C killing based on the release of ^{125}I from ^{125}IUdR prelabelled tumor cells has been developed.

Major Findings: The malignant guinea pig cells, line-1 and line-10, contain Forssman antigen. Line-10 sensitized with anti-Forssman antibody is resistant to lysis by guinea pig C (GPC) whereas line-1 is not, despite the fixation of equivalent quantities of anti-Forssman antibody.

An intermediate in the killing of line-1 tumor cells by antibody and human C (HuC) or GPC has been isolated. This intermediate, called T*, is a tumor cell coated with antibody and all the necessary components of C required for killing to occur. The formation of T* is dependent upon the time and temperature of incubation of antibody-coated cells with fluid phase C. T* were only formed on addition of the entire sequence of C components (1-9). A similar T* intermediate can be isolated in the antibody-HuC killing of line-10 cells.

The specific interaction of Concanavalin A (Con A) with IgM antibodies and staphylococcal Protein A with IgG antibodies was used to study the class of C-dependent cytotoxic antibodies produced after the immunization of rabbits with the antigenically distinct guinea pig hepatomas designated line-1 and line-10. Con A, either in the fluid phase or bound covalently to Sepharose 4B, inhibited C-dependent IgM cytotoxic antibody activity but had no effect on IgG activity. In contrast, Protein A inhibited IgG but not IgM. On the basis of this specificity, the antisera produced in rabbits to line-1 cells consistently proved to be a mixture of IgG and IgM antibodies, whereas the cytotoxic antibody produced against line-10 cells according to the same immunization schedule was exclusively IgG with the exception of one early bleeding in which some IgM activity was detectable. In all instances the class of antibody predicted on the basis of inhibition with Protein A or Con A was confirmed by the behavior of each antiserum in the fixation of the first component of C. ^{125}I labelled Protein A was utilized to measure the maximum number of IgG molecules bound to a line-10 cell. This value was of the order of 10^6 molecules/cell. Similar estimates of the number of cell-bound IgM molecules with the use of [^{125}I] Con A could not be made, inasmuch as unsensitized line-10 and line-1 cells bound approximately 1.1×10^6 and 8×10^5 molecules Con A/cell, respectively. Attempts are currently being made to develop an assay for the quantification of cell-bound IgM.

Studies on the resistance of line-10 cells to killing by antibody and GPC have revealed that this resistance could be overcome by pretreatment with a variety of proteolytic enzymes. This increase in sensitivity could not be attributed to the quantity of C-fixing antibody bound by the cells, or to a direct effect on fluid-phase C. Anticomplementary material was removed from the tumor cells by proteolytic enzyme treatment. This material was found to act as a C2 decay acceleration factor, but its removal was not solely responsible for the observed increase in sensitivity of the cells to antibody-GPC killing.

Significance to Biomedical Research and the Program of the Institute. The elucidation at the molecular level of the effects of antibody and C on both normal and malignant cells may be important in understanding control of cell growth by immunologic mechanisms.

Control of tumor cell growth in vitro makes it possible to study the action of specific and nonspecific agents on the cells at different stages of their growth cycle. The knowledge obtained from in vitro experiments will be very helpful when applied to the study of more complicated in vivo conditions.

Proposed Course of Project: The effect of antibody and C on nucleated cells will be further analyzed. This will include studies on the binding and utilization of C components during the cytotoxic reaction. The effects of the stages in the growth cycle of the cell and the physical and biochemical events that occur during formation and transformation of T* will also be studied.

Publications

Boyle, M. D. P. and Ohanian, S. H.: Quantitation of antibody-complement mediated lysis of tumor cells. J. Immunol. Methods 15: 135-146, 1977.

Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Effect of protease pretreatment on the sensitivity of tumor cells to antibody-GPC killing. Clin. Immunol. Immunopathol. 10: 84-94, 1978.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Complement-dependent cytotoxic antitumor antibody. I. Immunoglobulin class determined by interaction with Protein A or Concanavalin A. J. Natl. Cancer Inst. 60: 411-418, 1978.

Ohanian, S. H. and Borsos, T.: Killing of nucleated cells by antibody and complement. In Day, N. K. and Good, R. A. (Eds.): Biological Amplification Systems in Immunology. New York, Plenum Medical Book Co., pp. 115-135, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08554-08 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Isolation and Study of Tumor-Specific Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
OTHER:	Y. Keisari	Visiting Fellow	LIB NCI
	J. J. Langone	Senior Staff Fellow	LIB NCI

COOPERATING UNITS (if any)

None

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Humoral Immunity Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Identification, quantitation, isolation and characterization of tumor cell specific antigens are of importance in several areas of cancer research. Knowledge about these would aid cancer diagnosis, studies of the carcinogenic process, cell transformation, and cancer immunotherapy, among others. The present study concerns the isolation, purification and immunochemistry of soluble tumor antigens of transplantable guinea pig hepatomas.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To obtain tumor-specific antigens in soluble and partially purified form. To use these antigens in inducing, establishing and measuring tumor-specific immunity in guinea pigs. Knowledge derived from the guinea pig model will be applied to the isolation, purification and characterization of human tumor-specific antigens. These antigens will then be used in diagnostic studies of human tumor epidemiology, in monitoring patient response to treatment and as an aid in treatment. Initial studies will be on the ascites cell form of diethylnitrosamine-induced hepatoma of strain-2 guinea pigs.

Methods Employed: Source of antigen: ascitic fluid of tumor-bearing animals or extracts of cell surfaces with 3M KCl. Further purification: ammonium sulfate precipitation, gel filtration, DEAE chromatography. Assay methods: skin testing; in vitro correlates of cellular immune responses, immunodiffusion, immunization of guinea pigs with extracts and challenge of test animals with live tumor cells. Production and testing of heterologous antisera to tumor antigen: immunization of rabbits with whole tumor cells, absorption of sera with various cell suspensions, testing of absorbed sera by C1 fixation and transfer test, by immune fluorescence, and by cytotoxicity.

Major Findings: In order to obtain an enriched line-10 antigen preparation, the following procedures were employed: Absorption of ascitic fluid with affi-gel blue, which removes most of the albumin, followed by either high density ultracentrifugation or isoelectrofocusing (pH 3.5-10.0).

In both procedures a precipitation of the antigen occurred; thus soluble proteins could be further removed by high speed centrifugation (27,000 x g, 1 hr) or Sepharose 4B filtration.

Attempts to solubilize the precipitated antigen resulted in a severe loss of antigenic activity upon treatments with SDS, Deoxycholate, Triton X-100 or organic solvents, while 6 M urea and 3M KCl showed no effect on activity or solubility.

The antigen was stable to heat up to 56°C, and did not bind to Con A-Sepharose. Enriched line-10 antigen preparations elicited delayed hypersensitivity reactions in line-10 immune guinea pigs. Detection of antigen was facilitated by the development of a new assay for the antigen. Cell bound antigen can be assayed by the uptake of antigen specific IgG which in turn is detected by the uptake of ^{125}I Protein A. Soluble, cell bound or precipitated antigen can be detected by inhibiting the uptake by line-10 cells of the tumor-specific IgG. This method permits the use of formalized line-10 cells obviating the need for a daily supply of target cells needed for the cytotoxicity assay.

Significance to Biomedical Research and the Program of the Institute: The studies on the "naturally solubilized" antigens permit further characterization and immunochemical analysis of the line-10 and line-1 tumor antigens. Their presence in the tumor-bearing host and its immune response to them could offer one approach for a better understanding of the immune events in host-tumor interactions.

Proposed Course of Project: Further studies on the molecular nature of the soluble antigen and study of the biological significance of the presence of soluble antigens in the tumor-bearing host.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08555-06 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Detection of Complement Components on Nucleated Cell Surfaces

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. D. P. Boyle	Expert	LIB NCI
OTHER:	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI
	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	J. J. Langone	Senior Staff Fellow	LIB NCI
	A. P. Gee	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

Institut fur Immunologie der Universitat Heidelberg, Heidelberg, Germany

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Humoral Immunity Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.9	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop sensitive immunochemical methods for the quantitation of complement components on a molecular basis. The techniques will be applied to determine the amount and persistence of selected complement components bound to the cell membrane or tumor cells grown in vivo.

Project Description

Objectives: 1) To determine whether tumor cells of human (Hu) and guinea pig (GP) origin have detectable complement (C) components on their cell surfaces; 2) to develop techniques for the detection of C components bound to the cell surfaces.

Methods Employed: Immunochemical methods including DEAE chromatography, electrophoresis and immunodiffusion are employed to isolate antibodies, protein and C components. Quantitation of antibody and C components are carried out using radioisotopic labelling, quantitative precipitin procedures, C1FT tests, and immunochemical tests.

Major Findings: Methods have been developed to quantify C4, C3 and host immunoglobulins bound to the surface of tumor cells. These methods have been applied to two diethylnitrosamine-induced guinea pig hepatoma cell lines designated line-1 and line-10. The quantity of C3, C4 and immunoglobulin detected was dependent on the time of collection of the tumor cells (after intraperitoneal injection). C4, C3 and immunoglobulin were lost from the tumor cell surface during incubation in tissue culture. The loss of the cell-bound components occurs at 4°C and 37°C, with the greatest loss being at 37°C. The terminal stages of immune hemolysis have been studied to elucidate the action of the final component of the C sequence, C9. In these studies it has been shown that the release of cytoplasmic components from EAC1-8 occurs as a result of at least two sequential reactions following the interaction with C9. Binding of C9 to EAC1-8 occurred in a few minutes even at 0°C. Trypsinization of EAC1-9 prepared and held at low temperature resulted in nullification of the potential hemolysis of these cells. A brief incubation at 30°C or 37°C resulted in the formation of an intermediate whose hemolytic potential could not be nullified by trypsin. The failure of trypsin to nullify hemolysis was attributed to the insertion of C9 into the cell membrane. Studies on the effect of EDTA, or low temperature, suggested that the previously reported temperature-dependent step in E* formation was the insertion of C9.

The previous hope of developing a method for quantifying cell-bound C9, based on the ability of cells to interact with anti-C9 antibodies, now seems remote, since when C9 becomes inserted into the membrane it is probably no longer accessible for interaction with the antibody. Consequently attempts are being made to develop assays to measure the utilization of C9 from whole serum during the cytotoxic reaction. In addition, attempts are being made to purify C9 for radioiodination to facilitate the measurement of C9 uptake by antibody-sensitized cells.

Significance to Biomedical Research and the Program of the Institute: Techniques for detecting bound complement components may be useful in determining whether a humoral immune response has been produced to tumor cells in vivo. The ability to detect minute quantities of antibodies to tumor cells may provide means of relating humoral immune responses to antitumor effects in vivo.

Proposed Course of Project: Techniques for purifying C9 will be further developed and assays for utilization and uptake of C9, and other terminal C components, will be pursued. Attempts will be made to apply such techniques to determining whether resistance to antibody-C killing can be correlated with the quantity of later-acting C components bound to the cell. Additional studies concerning the fate of cell-bound immunoglobulin will be carried out using the existing methods. Radiolabelled Protein A will also be used as a probe to study 7S immunoglobulin bound to the tumor cell membrane.

Publications

Borsos, T. and Rother, U.: Deviated lysis: III. Kinetics of interaction of DL with chicken erythrocytes: Evidence for E* formation. Z. Immunitaetsforsch. 153: 36-47, 1977.

Boyle, M. D. P., Langone, J. J. and Borsos, T.: Studies on the terminal stages of immune hemolysis. III. Distinction between the insertion of C9 and the formation of a transmembrane channel. J. Immunol. 120: 1721-1725, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08556-02 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Ultrastructural Studies of Nucleated Cells Following Modification by Physical or Chemical Means

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Ohanian	Head, Electron Microscope Unit	LIB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Immunobiology, Immunology Program

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine what ultrastructural changes occur in tumor cells following modification with chemotherapeutic drugs, enzymes, hormones or following humoral immune attack. The techniques of Scanning Electron Microscopy and Transmission Microscopy will be employed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To determine the ultrastructural modifications occurring in tumor cells undergoing humoral immune attack, cellular immune attack and chemical or physical treatment which increases their sensitivity to immune attack; 2) to develop techniques for the localization of antibody and C binding sites on tumor cell surfaces.

Methods Employed: Antibodies to antigens of tumor cells and selected C components will be purified and labelled for detection in electron microscopy. Histochemical, immunohistochemical and immunoelectron microscopy techniques will be employed.

Major Findings: Procedures for the fixation of tumor cells in suspension or growing in the tissue of guinea pigs have been developed.

Tumor cells killed by humoral immune mechanisms appear enlarged and devoid of villi. Preliminary results of experiments with samples evaluated at different times after addition of C to antibody sensitized cells show a sequence of events in membrane perturbations. Local swelling or blebbing occurs at localized areas of the membrane followed by swelling of the cell and loss of villi. The surface of the cells killed by antibody plus C is extensively damaged compared to dead cells in control preparations.

Preliminary results of SEM examination of tumor cells growing in the skin and lymph node of guinea pigs indicate the morphology of the cell is similar to that of cells grown in the peritoneal cavity. Chemotherapeutic drugs injected into intradermal growing tumor cells induce minimal changes in surface morphology of the cells.

Attempts to prepare specific antibody labelled with hemocyanin or ferritin have not been successful. More refined methods will be employed.

The techniques of fluorescence polarization and electron spin resonance were used to examine the fluid properties of the membrane of cells treated with chemotherapeutic agents or hormones. In preliminary experiments no differences could be detected in the fluid properties of cell membranes using the electron spin resonance procedures. However, in fluorescence polarization studies using 1,6-D, phenyl 1,3,5 hexatriene, a probe which incorporates spontaneously into the lipid region of the cell membrane, differences were noted in the fluidity of the treated cells compared to control cells. Membranes of cells treated with actinomycin D and rendered susceptible to antibody-C mediated attack were more fluid than control cells. In contrast membranes of cells rendered resistant to killing by following pretreatment with hydrocortisone were less fluid than control cells.

Significance to Biomedical Research and the Program of the Institute: These studies are designed for analyzing the ultrastructural changes which occur in tumor cells following treatments which are effective in rendering the cells sensitive to immune attack. Visualization of physical attributes of tumor cells which may play a role in the resistance of tumor cells to immune attack should help in the understanding of defense mechanisms to immune attack.

Proposed Course of Project: Analytical studies of morphological changes occurring in tumor cells following antibody-C mediated attack will be expanded. Purified antibodies specific for Forssman and tumor antigens will be labelled with marker molecules for localization of these antigens on tumor cells. Characterization at the SEM and TEM level, of pathological features of tumor growth in vivo and of modifications following treatments with various chemical or physical agents.

Publications

Ohanian, S. H., Schlager, S. I. and Borsos, T.: Molecular interactions of cells with antibody and complement: Influence of metabolic and physical properties of the target on the outcome of humoral immune attack. In Inman, F. P. (Ed.): Contemporary Topics in Molecular Immunology. New York, Plenum Publishing Corp., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08575 06 LIB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. J. Leonard	Head, Immunopathology Section	LIB	NCI
	M. S. Meltzer	Surgeon	LIB	NCI
OTHER:	S. Watson	Visiting Fellow	LIB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Immunobiology, Immunology Program

SECTION

Immunopathology Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to study the cells that participate in the effector arm of the immune response. The current emphasis is on chemotaxis, which is a mechanism by which cells can be attracted to inflammatory sites, delayed hypersensitivity reactions and growing tumors. The project includes histology of different types of delayed hypersensitivity lesion, chemistry of lymphocyte derived chemotactic factors and identification of substances that modulate chemotactic and phagocytic responses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop quantitative measures of reactions occurring in the effector limb of the immune response; to study chemotaxis and phagocytosis of inflammatory cells in relation to this objective.

Methods Employed: Complement-derived and lymphocyte-derived chemotactic factors were generated. Human peripheral blood leukocytes were separated on Ficoll-Hypaque gradients to obtain monocyte- and basophil-rich fractions. Peritoneal macrophages were harvested from normal and BCG-infected mice. Protein fractionation methods were employed to characterize chemotactic factors and serum factors that modulate mouse macrophage chemotactic responses. Delayed hypersensitivity reactions were induced in guinea pigs. Histological study of the reactions was made in sections processed to preserve basophil morphology. Phagocytosis of antibody-coated erythrocytes was measured.

Major Findings: 1. Macrophage Stimulating Protein (MSP). It appears likely that physiological control of the mononuclear phagocyte system is mediated in part by humoral factors. One such factor, macrophage stimulating protein (MSP) was found in the serum of all mammals tested. It was assayed by its capacity to promote chemotaxis of mouse resident peritoneal macrophages to chemotactic stimuli such as endotoxin activated mouse serum. MSP itself is not a chemotactic stimulus. Further work on the characterization of this protein included development of a large scale fractionation schema suitable for 3 liters of human plasma, purification of MSP antigen approximately 2500-fold and production in rabbits of antibody that neutralizes MSP activity. Immunoabsorbent columns were made by reacting anti-MSP antiserum with protein-A-Sepharose. In addition to its chemotaxis-promoting effect, the MSP fraction also increased phagocytic activity and caused formation of long cytoplasmic processes. All 3 actions were abolished by anti-MSP antiserum. These 3 cellular activities involve movement of at least a portion of the macrophage and it is likely that the MSP fraction stimulates a rate-limiting step in a movement or contractility pathway. A potent SH compound, dithiothreitol, eliminated MSP-induced cell process formation and phagocytosis stimulation (effects on chemotaxis were not tested) but had not effect on unstimulated phagocytosis. DTT did not affect MSP binding. The results suggest that a disulfide compound plays a role in the MSP stimulation pathway.

2. Basophils. The mechanism of preferential localization of basophils in certain types of delayed cutaneous hypersensitivity lesions remains unknown. It was previously reported that culture fluids from PHA-stimulated human leukocytes increased the chemotactic response of basophils to complement C5a. This was of particular interest since among the various types of leukocytes the enhancement was restricted to basophils. A molecule of 35,000 molecular weight was isolated from purified PHA which increases basophil chemotaxis response to C5a or leukocyte-derived chemotactic factor. Antibody induced in rabbits by injection of PHA abolished both mitogenic and chemotaxis-enhancing effects of PHA. It also eliminated

the basophil chemotaxis augmentation effects of culture fluids from PHA-stimulated leukocytes, showing that the augmentation effect of culture fluids is due to a molecule in the PHA preparation and not to a product of stimulated leukocytes.

Significance to Biomedical Research and the Program of the Institute: The finding of a serum protein that stimulates cell process formation, chemotaxis and phagocytosis of resident mouse peritoneal macrophages shows that there may be regulatory serum factors that modulate macrophage motility. The role of the basophil in inflammation is only beginning to be understood; it is likely that secretion of its vasoactive mediators may play a critical role in the development of certain inflammatory responses, even of the delayed type.

Proposed Course of Project: Effect of MSP on various types of macrophages; measurement of MSP levels in serum and other body fluids of patients with cancer and other disease; characterization of serum factors affecting function of activated macrophages. Further study of basophil chemotaxis augmentation factor.

Publications

Leonard, E. J. and Skeel, A. H.: Isolation of macrophage stimulating protein (MSP) from human serum. Exp. Cell Res. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08576 06 LIB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunological Mechanisms of Tumor Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. J. Leonard	Head, Immunopathology Section	LIB	NCI
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COOPERATING UNITS (if any)

None

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is on the interaction of tumor cells and host defense cells. Current studies are on the depression of macrophage chemotaxis and increase in macrophage phagocytosis that occurs in experimental animals and on the increase in phagocytic response that occurs when serum from tumor-bearing mice is transferred to normal mice; mechanisms of macrophage activation and the impairment of activation in certain strains of mice; effects of lymphotoxin on radiation-damaged cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To determine what immunological mechanisms occur in tumor cell killing in order to provide a rational basis for elimination of tumor cells by immunological means.

Methods Employed: Chemically-induced murine tumors, maintained in tissue culture or by passage in syngeneic hosts were used. Effector immune responses were measured by (1) cytotoxicity assays based on the release of radioactive label from damaged target cells and (2) macrophage chemotactic response to complement or lymphocyte-derived chemotactic stimuli.

Major Findings: A. Macrophages. The progression of neoplastic disease depends on complex interactions between host and tumor. Mononuclear phagocytes play a central role in these interactions: macrophages have potent anti-tumor actions; tumor has profound effects on mononuclear phagocyte function. Macrophages with direct tumoricidal capacity, defined as activated macrophages, develop during a variety of immune reactions including tumor rejection. Development of activated macrophages requires the presence of an effective activation stimulus acting on subpopulations of mononuclear phagocytes responsive to the stimulus:

1. Macrophage activation factors are released from sensitized lymphocytes on contact with specific antigen. These factors are destroyed by heat (56°C for 30 minutes), by extremes of pH (below 4 and above 10) and by proteolytic enzymes. Chromatography of lymphokine-rich supernatants on the basis of size and charge suggests a limited size heterogeneity at a molecular weight of about 55,000 and an extensive charge heterogeneity suggesting the existence of more than one macrophage activation factor.

2. Quantitative analysis of lymphokine-induced macrophage activation showed that not all macrophages could be activated. Tumor cytotoxicity by peritoneal exudate macrophages activated in vitro by lymphokines was about 10-fold greater than that of equal numbers of lymphokine-treated resident peritoneal cells. This was due to an increased number of lymphokine-responsive cells in acute inflammation. They were peroxidase-positive and represented an influx of mononuclear phagocytes from the peripheral blood. The studies suggested that the lymphokine-responsive precursor of tumoricidal macrophages during both steady state conditions and acute inflammation was the immature blood-derived mononuclear phagocytes.

In certain situations, an activation stimulus acting on responsive mononuclear phagocytes was not sufficient for development of tumoricidal activity. C3H/HeJ mice, genetically unresponsive to the biological effects of bacterial lipopolysaccharide (unlike responsive C3H/HeN mice), failed to develop tumoricidal macrophages after intraperitoneal injection of viable M. bovis, strain BCG. Macrophages from these mice also did not become cytotoxic after in vitro treatment with macrophage activation factors present in lymphokine supernatants. The macrophage tumoricidal defect of C3H/HeJ mice is highly restricted: inflammatory responses to BCG infection in vivo or macrophage responses to chemotactic lymphokines in vitro were normal. Furthermore, production of macrophage activation factors by C3H/HeJ lymphocytes was normal. Further studies of these strains should throw additional light on the pathways for macrophage activation.

Macrophage function in mice with transplanted syngeneic fibrosarcomas was examined with 2 in vitro assays, chemotaxis and phagocytosis. In contrast to the previously reported persistent depression of chemotactic responses of resident peritoneal macrophages from these mice, phagocytosis of antibody-coated erythrocytes was markedly increased. Phagocytic capacity was 4-5 times normal within 1 week of tumor transplantation and returned to normal by 2 weeks. The increased phagocytic capacity, but not the chemotactic defect, could be transferred to normal mice by serum. The serum activity did not necessarily correlate with phagocytic capacity of the tumor-bearing donor: serum factors were detected for 6 weeks after tumor transplantation even at times when donor mice had normal phagocytosis.

B. Lymphotoxin. The release of tritium from thymidine-labeled target cells has been used as an assay of both cell and factor-mediated cytotoxicity. This assay is complicated by several findings: (1) The release of tritium is inhibited by undiluted cytotoxic culture fluids from stimulated spleen cells, despite visual evidence of extensive target cell destruction. Tritium release is also inhibited by sonicates of target cells and by concentrated conditioned medium from some lymphoid cell lines. (2) The tritium release is increased by incubation of the cells with DNase or trypsin. This occurred even when the enzyme was added for a 15 minute period at the end of the assay. Despite a large release of ^{3}H, there was no significant change in the number of target cells. Furthermore, by dye staining criteria, the remaining target cells before and after the 15 minute DNase treatment were viable and contained DNA. This puzzling finding is probably best explained by release from dead cells of not only soluble ^{3}H but also ^{3}H in compounds that adhere in an insoluble state to tissue culture plastic or viable cell surfaces until acted on by DNase or trypsin.

Factors that cause tritium release from target cells were produced by established cell lines as well as PHA-stimulated spleen cells. Culture fluid from 4 of 5 macrophage cell lines contained activity. In contrast, culture fluid from 12 T-cell lines, 9 B-cell lines and 1 mast cell line did not contain activity. The activity found in the culture fluid of one of the macrophage cell lines was located in 2 peaks on Sephadex G-200 chromatography. The position of the peaks was indistinguishable from two peaks found by G-200 chromatography of the culture fluid from a PHA-stimulated spleen cell preparation.

Significance to Biomedical Research and the Program of the Institute: Analysis of the effector immune events leading to tumor rejection may provide a rational basis for manipulation of host responses or tumor cell in an effort to eliminate or prevent progression of the tumor.

Proposed Course of Project: Studies on the mechanisms of the killing of tumor cells by macrophages; on the factors modulation macrophage chemotaxis and phagocytosis especially in tumor-bearing mice; further characterization of mouse lymphotoxin.

Publications

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Oppenheim, J. J., Mizell, S. B. and Meltzer, M. S.: Comparison of lymphocyte and mononuclear phagocyte-derived mitogenic amplification factors. In Cohen, S., Pick, E. and Oppenheim, J. J. (Eds.): *Biology of the Lymphokines*. New York, Academic Press, 1978.

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Annual Report of the Laboratory of Cell Biology

National Cancer Institute

October 1, 1977 - September 30, 1978

Mouse Myeloma Proteins

During the past year studies have continued of the primary and three dimensional structure of the antibody molecule. This project has involved the analysis of groups of antigen binding myeloma proteins which have been shown to have antigen binding activity for a series of chemically defined haptens including phosphorylcholine, galactan, and levan. The comparative study of the three dimensional structure of proteins with differing antigen binding specificities will permit an evaluation of the types of topographical changes which occur in antibody molecules to account for the structural basis of the vast degree of antibody diversity. The determination of the primary amino acid sequence of these and their naturally occurring counterparts, in addition to being essential for the three dimensional analysis, will further provide an insight into the genetic mechanisms involved in the generation of antibody diversity.

Previous studies on phosphorylcholine binding myeloma proteins have resulted in the crystallization and determination of the three dimensional structure of one of these proteins. These data combined with the primary amino acid sequence of the heavy chain permitted the construction of a molecular model of this antigen binding site. There has been recently completed the primary sequence of the light chain from this molecule. Crystallographic data are now in the final stages of refinement, and together with the new primary sequence data should in the near future produce a final molecular model defining the basic interactions occurring in this protein and in its interaction with antigen. Also now completed is the light chain sequence of a second phosphorylcholine binding protein and analysis continues of the patterns of diversity exhibited by these light chains in comparison to that of other kappa chains as well as previously reported data on the heavy chains from three of these proteins.

The germ line nature of the immune response to phosphorylcholine has been established by a variety of studies. The recent introduction of the hybridoma technique in which a plasma cell tumor line is fused with normal spleen cells and the resultant cell continues to make the product of the spleen cell but grows 'immortally' as a tumor, now permits us to look structurally at the products of a 'normal' B cell and compare these proteins to those found in the myeloma tumors. Hybridomas have currently been made to phosphorylcholine from both BALB/c and other strains. Analysis will be made on a series of these products to ascertain the pattern of variation, the relation to similar proteins from myeloma tumors, and the genetic polymorphism as reflected in the secreted immunoglobulins.

One of the longstanding questions in immunology is concerned with the basis of the generation of antibody diversity. It is still unclear as to whether the majority of diversity is encoded in separate germ line genes or if somatic mutation occurring on a relatively few germ line genes can account for the vast array of antibodies. While this laboratory and others have accumulated evidence for an increasing number of germ line genes, these two hypotheses are not mutually exclusive, and it seems quite likely that both participate in the generation of diversity. We are attempting to analyze the effect of a possible somatic mutation mechanism in the following system. Dr. M. Scharff has placed a line of a phosphorylcholine binding myeloma protein in long term tissue culture. Spontaneous mutants have arisen from this line which bind the antigen to a much lower extent but in other characteristics are identical to the parent line. A series of revertants and further mutants have subsequently been isolated from the original mutant. The hypothesis is that the variants are likely to result from single amino acid substitutions as would be expected to arise from a somatic mutation process. Structural analysis of these clones should establish the basis of the binding mutations. This phenomenon can also be analyzed in vivo by the isolation of hybridomas with characteristics of the parent line (idiotype) but decreased antigen binding capacity.

Another problem related to diversity currently being explored is that of the heterogeneity of the antibody response to protein antigens. To date very little is known about the structural basis of the immune response to protein antigens while these determinants along with carbohydrates are probably the most prevalent in nature. We are currently attempting to analyze the structural heterogeneity of the immune response to Staph. nuclease by isoelectric focusing. We have developed a transfer technique by which heavy and light chains are first separated and then electricfocused to analyse light chain heterogeneity. We are also attempting to develop a radioactive antigen overlay to assess heterogeneity of intact molecules and to evaluate polymorphism among inbred strains.

As mentioned above, proteins and carbohydrates are probably the most prevalent antigens in nature. We have also studied two groups of myeloma proteins with binding activity for the carbohydrates β(2→1) levan and β(1→6) galactan. Details of the anti-levan studies were presented in the preceding Annual Report. Analysis of light chains from four galactan binding myeloma proteins has established that this group of molecules must be coded for by at least one germ line gene and the variation among these light chains is minimal as in the case of the kappa chains from levan binding myeloma proteins. Evidence also suggests the probability of multiple genes coding for at least part of the constant region.

The heavy chains from the galactan binding myeloma proteins have presented difficult problems in terms of peptide isolation. Experimental procedures have been developed in the last year which have now permitted isolation of the appropriate fragments. Nearly complete sequences are available for three heavy chains from this group and a fourth is currently in progress. The completion of the galactan sequences will be the final segment of our study on groups of antigen binding myeloma proteins. At this point we are now analyzing our accumulated data from phosphorylcholine, levan, and galactan binding proteins in terms of the compatability of the patterns of variation observed with the mechanisms proposed for generation of antibody diversity. The galactan binding proteins are of further importance in that two of these have been crystallized and X-ray crystallographic studies are currently in progress on these molecules. The X-ray data combined with the primary sequence analysis will permit construction of a molecular model which can then be compared with the previously determined structure of the phosphorylcholine binding protein M603.

Studies of Immunoglobulins

Comparison of the amino acid sequences of many rabbit light chains have revealed two segments of hypervariability which correspond to the first and third hypervariable regions found in the light chains of other species (residues 24-34 and 89-97). These regions are involved in forming the antigen binding site and are termed complementarity-determining regions. Elicited rabbit antibodies to type III and type VIII have markedly different hypervariable sequences and lengths.

In a partially inbred colony 6 light chains from elicited antibodies to streptococcal group A variant carbohydrate have been reported with identical sequences of the first hypervariable region. We have studied a light chain from a rabbit antibody to type III polysaccharide with a sequence strikingly similar to the light chain from anti-A variant antibodies and therefore belonging to the same subgroup as the previous light chains. More interesting was the finding that residue positions in the first hypervariable region were identical. The major differences between the two types of chains were found in the third hypervariable region, although they were identical in length. Several differences were also found outside the hypervariable regions, indicating that this single subgroup is represented by more than one germ-line gene. The question arises why light chains of anti-type III and anti-A antibodies of unrelated rabbits share the first hypervariable region and yet are very different in the third hypervariable region. One likely possibility is that the first hypervariable region is not participating in antigen binding, in either case; however, more work such as heterologous chain recombination and determination of the binding capacity for either antigens required to assess correctly the significance of our finding.

Comparison of the complete amino acid sequence of the shortened heavy (α) chain of mouse IgA47 to that of a human chain revealed that the entire C_H3 domain of the 47A α chain had been deleted and that the hinge region differed both in length (seven residues shorter) and sequence. The sequence of a non-deleted mouse α chain (MOPC 511) has been undertaken in order to provide a more meaningful comparison to the deleted 47A chain. The data obtained thus far show that the hinge regions of the two mouse α chains are identical and that the C_H1 and C_H2 domains are probably identical except for a single interchange of threonine to alanine. Cys-135 of 47A which participates in the light-heavy chain band is also present in the 511 λ chain despite the fact that the chains of 511 are not covalently linked. It has been tentatively concluded that IgA 47A is a mutated allotype of IgA 511. The absence of C_H3 domain in 47A apparently induces a profound conformational change which not only presents the formation of a four-chain molecule, as in 511, but allows the formation of a light-heavy disulfide bond.

One of the continuing efforts of this laboratory has been to systematize the V-kappa isotypes of the BALB/c mouse. Fortuitously the amino acid sequence of the chains up to the first invariant Trp35 provides a means for typing and identifying germ line V_k-structural genes. The mouse has a great diversity of structures (33 V-kappa isotypes so far identified) which are expressed in the myeloma pool and many of these have been related to specific antibodies.

We continue to study V_k-isotypes by partial sequencing, 25 new kappa chains through Trp35. These data hopefully will define the extent of V-kappa genome in the BALB/c mouse. The mouse is an ideal species for studying kappa chain diversity because, for evolutionary reasons not understood, the framework region, FM-1, has many differences from one V-region to another. This is in contrast to human V_k in that only 4 to 5 FM-1 subgroups have been defined. We may therefore be able to estimate the V-region genome size in the mouse by primarily structural data.

In collaboration with Dr. D. J. McKean of the Mayo Clinic we continue to determine sequences on the VK-21 group of proteins. This group now contains 5 isotypes VK-21A to E. VK-21 isotypes may differ from each other by as few as 5 positions. Since an isotype is a minimal definition of a germ line V-gene, the BALB/c mouse genome contains a large number of V-identifiable V-K genes. The genetics of Vk genes offers exciting opportunities in the area of mammalian gene regulation. There is a great need to develop Vk markers in the mouse based upon homologous Vk differences. We hope to identify such markers using wild mouse genes from species of musculus e.g. M. m. molossinus and M. m. castuneus. Hybridomas of molossinus and castuneus with MOPC21 (P3) are to be made by S. Clarke, in collaboration with F. Melchers at the Basel Institute and these will be compared with homologues in BALB/c as a potential source of V_k markers.

Studies continue of the β1,6 galactan binding myeloma proteins (GALBMP) and their relationship to β1,6 galactan antibodies. These proteins all have VK1B-Vk4 isotype composition. We have induced antibodies to gum ghatti in mice and shown these antibodies share cross-specific idiotypes (IdX) with GALBMP. Each of the 8 GALBMP however, has a characteristic private or individual idiotype (IdI). BALB/c antibody by RIA analysis contains a large amount of XRPC24 IdI indicating the XRPC24 may be very closely related to the germ line gene. The regulation of the VH1B-Vk4 expression will be further explored this year.

Histocompatibility (H-2) Antigens

The heavy chains of papain-solubilized $H\text{-}2^a$ antigens ($H\text{-}2K^k$ plus $H\text{-}2D^d$) have been purified to homogeneity and chemically characterized. The molecules consist of 290 amino acid residues, contain two disulfide bridges and one free cysteine residue, and have homologus NH_2- and COOH-terminal sequences. The NH_2-terminal sequences of the H-2 chains closely resemble those of HLA. However, the solubilized mouse and human histocompatibility antigens differ in chain length, COOH-terminal sequence, and cysteine content. These observations suggest that papain cleaves membrane bound H-2 and HLA at different positions with respect to the COOH-terminus.

A serum component that appears to be shed H-2 antigen has been purified from serum of Swiss BALB/c mice strain. It represents the major β_2-microglobulin bound component in serum and contains in addition to β_2, another polypeptide of approximately 38,000 daltons. The larger polypeptide chain has an amino acid composition closely related to the large peptide of H-2 antigen solubilized by papain. It lacks alloantigenic specificities but shares with H-2 xenoantigenic determinants detected by a rabbit antiserum raised against the shed, H-2. This molecule could represent an H-2 which is negative for alloantigenic specificities but still retains the basic structure of H-2.

Tumor Associated and Tumor Specific Antigens

Meth A Antigens

We have returned now to attempts at purification of the individually distinct TSTA on Meth A sarcoma cells. Previously we had achieved good isolation but with only minimal purification over the specific activity of the crude membrane (CM) preparation. Meth A has certain advantages over other systems: a) the neoplasm exists in ascites form, b) it has extremely strong TSTA, c) it has no detectable alien H-2 specificities and d) the *in vivo* passaged line has no infectious C type virus or virus fingerprints. In addition Dr. Old's group at Memorial-Sloan Kettering have developed a tumor-specific antibody against Meth A and hopefully a competition-binding assay can be developed to aid in assaying fractions during purification; we are cooperating with the SK group in this aspect of the study.

We have found in this new study that purified membranes solubilized with NP40 and chromatographed on AcA22 columns that extremely small amounts from Fraction III, subsequently fractionated on Lens culinaris lectin and wheat germ agglutinin columns (to completely remove H-2 and β_2-microglobulin, retained specific tumor rejection activity against Meth A; 95% tumor inhibition was achieved with as little as 1.0 μg protein. AcA54 chromatography following lectin fractionation has resulted in a major peak (60,000 daltons) of TSTA activity. This material gave only 1 band on SDS gels. It is our feeling that purification will be greatly enhanced now beginning with the 60,000 dalton material from AcA54 gel filtration and work is continuing with this material.

All biologic assays we have completed with our solubilized, partially purified material indicates activity similar to that observed using intact Meth A cells - thus the antigen is not degraded, specific immunogenicity is maintained, there was observed no "immune deviations" etc.

SV40 Tumor Antigens

Extensive cross-reactivity studies have been reported for the nuclear T antigen: antigenically related T-ag have been demonstrated in SV40 transformed cells from different species and those induced by the variant viruses recovered from man: DAR, BK and JC. We have previously reported cross-reactivities of TSTA of SV40-induced neoplasms of several species, man, rat, sheep, hamster, mouse etc. employing the mKSA system in BALB/c mice. Intact cells, plasma membranes and solubilized TSTA were equally effective. When, however, we employed the immunosensitive SV34 tumor of the hamster we found that whereas all SV40-, BK- and JC-induced neoplasms, membranes and solubilized ag immunized the mouse only SV40 virus and SV34 intact cells (not membranes nor soluble antigen) were capable of immunizing the hamster. These results strongly suggest the existence of different species of SV40-encoded TSTA. We have developed a concept to explain these findings in terms of different species of TSTA in the mouse and hamster. (See Law, Takemoto, Rogers, Henriksen and Ting, Int. J. Cancer 1978, in press).

More recently and equally interesting are the findings that neoplasms induced in the hamster by SV40, BK and JC viruses respectively have their own individually distinct tumor rejection antigens (TSTA) that do not cross-react. It has been reported however, that the T-antigens of these neoplasms are antigenically related.

We have found also that the TSTA from SV40-induced neoplasms of the mouse (mKSA) can be obtained simply by homogenization of dissociated cells and gel filtration without resort to solubilization with detergents. This H_2O soluble antigen retains all the biologic and immunogenic characteristics of the intact cell: immunization is achieved easily with a single dose of 10^6 cell equivalents, this immunization is specific for SV-40 induced neoplasms, the immunity provided by soluble antigen is stable and long lasting (150 days), there is no "overloading" with large doses of antigen. This soluble SV40 antigen is now being used in attempts to induce secondary cytolytic T lymphocytes in vitro. A more detailed understanding of molecular events involved in recognition in a syngeneic tumor-host system (as opposed to allogeneic systems) is therefore now possible.

TAA on FMR-MuLV-induced Leukemias

A clearer picture of the tumor associated antigens (TAA) present on plasma membranes of leukemias induced by the FMR-MuLVs is now emerging as a result of studies of the biologic activities of the different molecular entities obtained by solubilizing with Na-DOC and chromatographing using AcA34 and Lens culinaris lectin affinity columns. The following findings are pertinent: a) Tumor rejection activity (TSTA) is solubilized and partially purified (based on yields of specific activity) and is found principally in high MW fractions (2, 3 and 4 of the AcA34 column), b) TSTA activity is clearly separated from H-2, gp70, p30 and p15E activity by gel filtration, c) The partially purified TSTA retains the specificity of the intact cells - providing tumor rejection against FMR leukemias but not against spontaneous and MCA-induced leukemias - nor against other neoplasms, d) A reasonable dose-response is observed at lower doses (from 1 μg to 25 μg) but interestingly doses of antigen (membranes as well as solubilized materials) greater than 25 μg do not provide rejection of RBL-5. The reasons for the induction of this "null" state is presently under investigation, e) A second antigen, unrelated to TSTA, and detected by in vitro cytotoxicity against RBL-5 employing syngeneic antisera, has been identified. This is a low MW entity (fraction 9 from the AcA34 column) and appears to be p15E. This antigen is unlike the TAA described by Fenyö and Klein present on Moloney virus-induced TAC cells and unlike the antigen on Moloney virus induced leukemia cells described by Ng; this latter appears to be related to viral structural proteins.

The purification of TSTA is being continued by preparing syngeneic and xenogeneic antisera against the most highly purified fractions to be used as immunoabsorbants. $H-2^b$ from RBL-5 membranes has been isolated, solubilized and purified more than 1000 fold (50% pure) and now obtained in quantities suitable for structural characterization.

Characterization of Alien H-2 Molecules

H-2 antigens in a modified form or as derepressed antigens have been implicated indirectly by several investigators in host immune responses to tumors - specifically evidence points strongly to H antigens, in some form, to be tumor specific antigens (of the transplantation type). We are looking for such evidence in several systems:

Since alien (or inappropriate) H-2 specificities have been described in several methylcholanthrene-induced sarcomas in BALB/c mice, particularly by Parmiani and Invernizzi of Milan, we have studied a series of 7 MCA-induced neoplasms of BALB/c (from pedigreed lines to assure non-contamination). Through the use of *in vivo* immunogenicity assays - growth and the induction of immunity in various F_1 hybrids with BALB/c, we have found no evidence for inappropriate or alien H-2 determinants and in limited studies using serologic assays, no evidence was found for any H-2 determinants except those public and private specificities determined by $H-2D^d$ and $H-2K^d$ loci. Thus, all of these sarcomas do have individually distinct TSTA and at least no detectable modified or derepressed H-2 antigens appear to be functional as TSTA.

Studies continue concerning characterizing the set of alien specificities (H-2, 1, 5, 11, 23 and 25) representing the $H-2K^k$ haplotype on Parmiani's C-1 MCA-induced sarcoma. Two questions of major interest are, 1) does this molecule act as the tumor specific transplantation antigen (TSTA) of the tumor - as claimed on indirect evidence and 2) is the molecule the same or different from the normal $H-2K^k$ molecule (e.g. from C_3H cells)? The following pertinent facts are now known comparing the normal and alien H-2 antigens from purified membranes of C-1 cells: 1) The molecular weight of the molecule bearing alien specificities is 50% greater (150,000 daltons) than the normal (100,000) in gel filtration; both are glycoproteins. 2) The normal antigen, as most H-2 heavy chains, binds β_2-microglobulin tightly whereas there is no binding or very loose binding with the alien antigens. 3) There is very little of the alien antigens on the cell (as much as 100X less) compared to normal H-2; this of course could reflect differences in binding, 4) Whereas normal H-2 antigens resist papain digestion, the alien antigens are very sensitive - and most may even be destroyed by papain. Although the specificities as detected serologically are all present on the C-1 molecule and appear similar to normal $H-2K^k$, the above results tend to indicate that the "alien" specificities and normal specificities are on different molecules. One additional biologic difference is reflected in the C-1 alien antigens inability to evoke a cellular response - contrary to the normal antigens. Work is progressing on the purification of the alien antigen to compare it with H-2 and TSTA and to determine in a more definitive way if this alien antigen is a tumor specific antigen. So far a 20 fold purification has been attained.

Viral Oncogenesis and Viral Gene Expression

We continue to study the role of the defective Abelson retrovirus as an initiator in plasmacytomagenesis. In collaborative studies with Dr. Charles Scher of the Sidney Farber Cancer Center, Boston, Ma., we are studying the plasmacytomagenic properties of MLV-A in vitro. Cells from CB-20 mice are infected with MLV-A and stimulated with LPS. After varying intervals the cells are transplanted into BALB/c mice. The myeloma proteins produced by to induce one tumor in 8 days in culture. This method is very promising and could lead to the identification of the MLV-A target cell type. We shall explore Peyer's patch, peritoneal exudate, mesenteric node and spleen cells as a source.

In collaborative studies begun with R. Lerner (Scripps Institute) and continuing with J. Hartley (NIAID) type C viruses have been isolated from plasmacytomas in early transfer generations including one duotropic MCF type virus, and several N- and B ecotropic. Early transfer generation plasmacytomas have been successfully superinfected with Moloney virus (the rescuing agent for MLV-A) (in collaboration with C. Scher) extracts from these tumors currently being studied. Early developing leuekmias will be studied for the presence of transforming viruses.

In collaboration with J. Hartley (NIAID) we are testing the duotropic MCF (mink cell focus forming) retrovirus for plasmacytomagenesis activity both by the in vitro infection method, and by direct in vivo inoculation.

Adherent peritoneal exudate cells, from pristane primed BALB/c mice are being systematically studied for retrovirus production. Viruses can be isolated from adherent peritoneal cells with in the first few weeks after pristane. Adherent cells are being studied from mice in the pre-plasmacytomagenic period and, the types of viruses obtained from adherent cells will be compared with those obtained from the primary plasmacytomas that develop in these mice.

Intracisternal A particles (IAP), Retroviridae, are distinctive in that they are confined to cytoplasmic cisternae of various tumor cells (myeloma, mammary tumor, neuroblastoma, rhabdomyosarcoma, and yolk sac carcinoma), embryonic cells, and rarely in normal adult cells. When isolated and purified from murine tumors, they have never been shown to be infective. We have in the past cultivated mouse cell lines, which were negative for the IAP phenotype, with whole cells, subcellular fractions and purified IAP without successful stimulation of IAP production in these negative recipient cells. The lack of infectivity of isolated, purified IAP may reflect impaired entry and uncoating within the host cell; additionally, the potential infective capacity of IAP is significantly compromised by an isolation procedure which requires detergent treatment. Until now, horizontal transfer of IAP could only be demonstrated by fusion of IAP-positive cytoplasts, derived from mammary tumor cells (MT), with IAP-negative recipient cells. In this model for horizontal transfer of the IAP genome, we have used as the IAP donor, a continuous cell line established in this laboratory and derived from a BALB/c mammary tumor. As recipients, two phenotypically negative cell lines were used, 3T3-4EF cells (NIH Swiss) and the II6C clone of SC-1 cells (feral). Mammary tumor whole cells or cytoplasts (enucleated cells) were fused to these lines. The resulting hybrids and cybrids have previously been well characterized. Using a radioactive cDNA probe synthesized by the reverse transcriptase reaction of MT IAP for DNA-DNA hybridizations, we have approximated the number of copies of IAP-specific DNA sequences in the parent cells and their related hybrids and cybrids. The purpose of this study is to define more precisely the mechanisms of IAP phenotypic transfer and in particular to determine whether IAP-specific DNA sequences already exist in these recipient cells or whether such DNA sequences are integrated *de novo* as a result of the fusion process.

Based on the copy numbers of IAP-specific DNA sequences observed in the various parent cells, hybrids, and cybrids, and the thermal stability characteristics of the DNA duplexes, two mechanisms were proposed for IAP expression in the cybrid cells which were formed by the fusion of IAP-positive cytoplasts with IAP-negative cells. First, in the case of a genotypically negative recipient cell, 3T3-4EF, fused with the IAP donor MT cytoplast (cybrid clone J18) the copy numbers of IAP-specific DNA sequences increased from 1 to 10. The horizontal transfer is probably mediated by a reverse transcription of the IAP genome, followed by subsequent *de novo* integration and transcription of the DNA sequences, thus accounting for the continuous replication of IAP in the cybrid cell. By cell fractionation, we separately isolated and purified the cytoplasmic DNA from the nuclear DNA derived from five grams of J18 cybrid cells. We found that the *de novo* integrated IAP-specific DNA sequences all resided in the nuclear DNA fraction, implicating that such sequences have been permanently integrated into the cell genome.

Second, in the case of the D4 cybrid cells, in which II6C, the phenotypically IAP-negative feral mouse cell, already carries the IAP genome, IAP transfer by cybridization with the MT cytoplast did not increase the number of IAP-specific sequences. The IAP expression in the D4 cybrid cells may be explained by the derepression of a regulator gene controlling the expression of IAP in II6C, due to a factor transferred from the MT cytoplast. It seems therefore the expression of IAP also comes under the control of a regulator gene.

In an ongoing project the GAG and ENV gene products of two rat viral complexes were exhaustively analyzed by biochemical and radioimmunological techniques. One of the rat viral complex, KiMSV(RHHV) was isolated in this laboratory. By equillibrium polyacrylamide gel electrophoresis and fluorography, we have found that both viruses, when their proteins were chronically labelled with either ^{35}S-methionine or ^{3}H-amino acid mixture and isolated by immune precipitation with anti-RaLV serum, possess four proteins at variant amounts: these are 110-120,000 mol. wt. (P110-120), 70,000 mol. wt. (P70), 30,000 mol. wt. (P30), and 7-10,000 mol. wt. (P7-10). Among these proteins, the P70 and P110-120 are both glycosylated since radioactive glucosamine or galactosamine were both incorporated into the same viral protein peaks. Other glycosylated proteins associated with the virus are the 180,000 mol. wt., gp180, the 85,000 mol. wt., gp85, and the 40-45,000 mol. wt., gp40-45. Some of these large mol. wt. proteins or glycoproteins, gp180, P110-120 and gp85, were of cellular origin that were precipitated by the anti-RaLV serum during immune precipitation. That they may be precursors of the low mol. wt. structural proteins or glycoproteins has been investigated by pulse-chase experiments. The nature of the small mol. wt. P7-10 is not yet known.

Intracisternal A particles

A collaborative study with Dr. Raymond Gantt has yielded additional information about certain events which occur during the formation of intracisternal A particles (IAP). Since it had previously been shown that several murine and avian retroviruses had an associated N^2-guanine RNA methylase, and since IAP exhibit several characteristics of retroviruses, it seemed pertinent to determine if such an enzyme was associated with IAP. The results of this investigation indicated that six methylases were associated with IAP, a cytidine and a uridine methylase, two N^2-guanine monomethylases, an N^2, N^2-dimethyl-guanine methylase, and an adenine methylase. Using *E. Coli* phenylalanine tRNA as an acceptor RNA, these enzymes were shown to be indistinguishable from cellular enzymes. However, it is of significance that the specific activity of these enzymes increased about two fold when IAP cores were prepared by stripping the outer envelopes with 1% Triton X-100; this result strongly suggests that the methylases are present inside the particles and not just absorbed to the surface. It seems probable that these cellular enzymes are packaged within IAP during the process of synthesis. The function of such RNA emthylases is not known, but it is of interest that an analogous situation exists in the case of Rous sarcoma virus and avian myeloblastosis virus wherein cellular tryptophan tRNA is intimately associated with the 70S RNA and can serve as an effective *in vitro* primer of DNA synthesis.

There is abundant evidence that intracisternal A particles are not released from cells. Thus it is not surprising that a variety of cell cocultivation experiments have failed to demonstrate successful horizontal transmission. This failure of release can be abrogated in a least two ways. One, IAP-containing cells can be fused to particle-negative cells, in the presence of inactivated Sendai virus, and IAP are expressed as a dmoninant genetic trait in the resulting hybrids. Two, cytoplasts (enucleated cells) can be used as carriers of the IAP phenotype and when fused to particle-negative whole cells, the resulting cybrids contain IAP which are not segregated over long term passage. Therefore a form of horizontal transmission has been achieved. We have recently completed studies which more precisely define the nature of the infectivity associated with IAP. Using the endogenous reverse transcriptase associated with IAP, it has been possible to generate a cDNA probe and determine the approximate number of gene copies in the parent cells, the hybrids and the cybrids used in the fusion experiments. It was initially established that there was homology between cDNA probes derived from IAP purified from either plasma cell tumors or mammary tumors. Mammary tumor cells were used as IAP donors and two particle-negative lines, 3T3-4EF (NIH Swiss) and II6C (feral) were used as recipient cells. The MT cells contained 20-24 copies of IAP-specific DNA sequences per haploid genome and the hybrid cells had 1 or less IAP copies per haploid genome and the II6C cells had about 4 copies per haploid genome. Therefore two mechanisms can be invoked to explain the observed results. In the case of the MT-II6C cybrids, the presence of IAP suggest that a derepressor in the MT cytoplasts may have switched on the pre-existing IAP genes in the II6C cells. In the case of the MT-3T3-4EF cybrids, which contained 15-20 copies per haploid genome, the presence of IAP may well reflect the possibility that the IAP genome was transferred from the MT cytoplasts to the 3T3-4EF DNA by reverse transcription, with subsequent integration and production of particles. Such a sequence of events suggests an analogy to the replication of retroviruses.

Xenotropic Viruses

Since previous experiments with an ecotropic virus (designated D1-MuLV) isolated from SJL/J mice did not give conclusive evidence for its direct role in the etiology of RCN, the possibility of an involvement of xenotropic virus in this system was examined. Studies in AKR mouse indicated that an increased activity of ecotropic virus which by itself is not leukemogenic may lead to activation of xenotropic virus and manifestation of leukemia, with concomitant production of dual tropic, recombinant viruses. Although no such dual tropic virus has been isolated from SJL/J mice so far, the presence of xenotropic virus in the normal SJL/J mouse embryo and RCN (designated SJL/J-MEF-X-MuLV and SJL/J-RCN-X-MuLV respectively) suggests that the above mechanism of oncogenesis may be operative in SJL/J system. Toward this end, the xenotropic viruses isolated in this laboratory were first characterized by the following criteria.

a). Host range: By measuring at weekly intervals for 4 weeks the reverse transcriptase activity of virus pellets obtained from the culture fluid of infected cells, the following cells were found to be permissive for replication of both SJL/J-MEF-X-MuLV and SJL/J-RCN-X-MuLV: Human embryonic fibroblasts, human skin fibroblasts, a human osteosarcoma cell line, and a human conjunctiva cell line (WK-D); a mink lung cell line (Mv 1 Lu); a rabbit cornea cell line (SIRC); feline embryo fibroblasts; rat embryo fibroblasts and NRK (normal rat kidney) cell line. The following cells did not support the replication of these viruses. NIH-Swiss, BALB/c and C57BL/Ka mouse embryo cells; a wild mouse embryo cell line (SC-1); hamster embryo cells and BHK-21 cell line; a monkey kidney cell line (LLC-MK2); chick embryo cells. These results were essentially similar to those obtained with two other xenotropic viruses, AT124 virus isolated from NIH-Swiss mouse, and NZB virus isolated from NZB mouse embryos.

b). Template and ion preference in reverse transcriptase assays: Like other type-C viruses, these xenotropic viruses exhibited a preference for Mn^{2+} ion over Mg^{2+} ion when oligo (dT).poly (rA) was used as template. Oligo (dA).poly(rU) was less efficient (1/10) than oligo (dT).poly (rA) in the presence of Mn^{2+} for SJL/J-X-MuLV's, and AT124 virus, but these two templates were equally efficient for NZB-X-MuLV. In the presence of Mg^{2+}, however, oligo (dT).poly (rA) was inactive for all of these viruses tested, but oligo (dA).poly (rU) were moderately active for SJL/J-X-MuLV's and AT124, and much more active for NZB-X-MuLV. Therefore, SJL/J-X-MuLV's behaved more like AT124 than NZB-X-MuLV in these respects.

c). Viral interference patterns: SIRC cells preinfected with SJL/J-MEF-X-MuLV, SJL/J-RCN-X-MuLV, AT124, or NZB-X-MuLV were challenged with respective MSV pseudotypes and the resulting infectious centers (cells producing MSV pseudotypes) were assayed by plating the Mitomycin-C-treated SIRC cells on NRK cells. Such a two-step test was chosen because, although SIRC cells were more sensitive than NRK, the transformed morphology of SIRC cells made it difficult to assess the number of transformed MSV foci. The results indicated that there were reciprocal cross-interference between these viruses, suggesting that these viruses belong to the same class of X-MuLV's.

d). Virus neutralization tests: Rabbit antisera against these X-MuLV's were prepared and tested against MSV pseudotypes of these X-MuLV's for inhibition of MSV focus formation in NRK cells. In general, there were cross-neutralizations among these viruses, indicating their close antigenic relationships within the same class of X-MuLV's. However, there was a slight difference between AT124 and NZB-X-MuLV, and the SJL/J-X-MuLV's behaved more like AT124 than NZB-X-MuLV in the neutralization test. Fischer rat antiserum against ecotropic viruses, Dl-MuLV (from SJL/J mouse), Moloney-MuLV, and AKR-MuLV were also tested along with the rabbit anti-X-MuLV sera in cross-neutralization tests. It was found that there was a certain degree of sharing of envelope antigens between SJL/J-X-MuLV's and Dl-MuLV indicating the possibility of phenotypic mixing or recombinational events occurring in the SJL/J mice.

e). Further tests for comparisons of p12 antigenic differences among these viruses and BALB:virus-2 by competitive radioimmunoassay are in progress. These tests would indicate whether SJL/J-X-MuLV's are more related to one subclass of X-MuLV than to another, and help to elucidate the evolutional origin of these viruses. Further work performed to determine the frequency of isolation of X-MuLV's from normal spleens of SJL/J mice at different ages, i.e. prenatal, 3 weeks, 6 weeks, 3 months, 6 months, 9 months, and 12 months. The preliminary results indicated that there were more X-MuLV's at 6 and 12 months of age than at other ages, suggesting that the X-MuLV's are associated with higher frequency of spontaneous RCN occurring in the older age group.

Molecular Genetics of Mammalian Mitochondria

In the past year we have extended our experimental work showing that chloramphenicol resistance (CAP-r) could be transmitted to chloramphenicol sensitive (CAP-s) cells by treatment with purified mt-DNA alone. We have tried to decide if the mechanism of this "transformation" event was by 1) repopulation of the whole CAP-s cells mt-DNA by the entire CAP-r molecule, or 2) by a recombination-like event (similar to the incorporation and stabilization of antibiotic resistance markers in *Pneumococcus*). We have used subspecies of the laboratory mouse, *Mus musculus musculus*, and *M. m. molossinus*, and a closely related species *Mus caroli*. All of these mt-DNA molecules are easily distinguished using restriction endonuclease mapping. In short, our results have shown that in 4 out of 4 transformed cell strains examined, there was no evidence of persistent CAP-r mt-DNA still present in the treated cell lines. This result implies strongly that a recombination-like mechanism supersedes when CAP-r mt-DNA of one subspecies is used to "rescue" a CAP-s cell strain of another subspecies. Our first efforts at examining these transformants at higher resolution (endonucleases yielding 27-35 fragments from a single mt-DNA molecule) for evidence of changed restriction sites has shown that one of the four examined has at least one changed restriction site. We have also established during the past year that all of the "transformants" are themselves capable of transmitting the CAP-r phenotype by cytoplast fusion and hence they do represent changes in the cytoplasmic markers.

Parallel to these studies of the mt-DNA mediated transformation we have initiated cybrid and hybrid experiments between the same sets of subspecies examined in the previous experiments. One of these KYK (*M. m. molossinus*) a CAP-s cell line that has received the cytoplasts of OC10, a CAP-r, *M. m. musculus* cell strain has been found surprisingly to show 50% *molossinus* mt-DNA and 50% *musculus* mt-DNA after 40-60 cell generations of CAP selection. This result was surprising because we had believed that the two subspecies were fully compatible as far as the mitochondria were concerned. Now, however, at equilibrium under CAP selection it is apparent that KYK (*molossinus*) cannot live with *musculus* (OC10) mt-DNA alone; it must preserve its own kind for some mysterious reason. Other evidence suggests that in this particular cell strain we have encountered an interdependence of mt-DNA functions that may lead us to the first nuclear genes to be found that are involved in the reglation of mt-DNA synthesis.

Although there are several purely genetic experiments that are being explored with these heterosubspecific cybrid cell lines that would be too involved to chronicle here, the cell lines may allow us to make a very convincing demonstration of the fact that CAP-r and mt-DNA segregate together (are indeed conincident). Our experiments have shown us that when such cybrids are cultivated for a long time in the absence of CAP, the selective agent, then the *musculus* CAP-r mt-DNA molecules may be lost from many subclones in the population. Such segregants have also lost the CAP-r phenotype. In cases where the cells have retained the CAP-r phenotype they also have the *musculus* mt-DNA. This kind of syntenic segregation analysis is used extensively to establish chromosomes assignments in the human karyotype using human-mouse segregating hybrids.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03200-09 LCBGY

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
A Study of the Various Factors Influencing the Induction, Growth and Repression of Primary and Transplantable Neoplasms (Host and Tumor Cell Variables)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. W. Law	Chief	LCBGY	NCI
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LAB/BRANCH
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TOTAL MANYEARS: 6.50	PROFESSIONAL: 2.00	OTHER: 4.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: Major emphasis is placed upon the study of tumor antigens of the transplantation rejection type (TSTA) and of the immune responses they evoke. As a corollary to this study the biologic properties in vitro and in vivo of Histocompatibility (H-2) antigens are under study. Solubilization and methods of purification of both TSTA and H-2 are under investigation with the ultimate purpose of defining these membrane antigens in physico-chemical, biologic and molecular terms.

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I. Histocompatibility Antigens

1. After several years H-2 antigens have now been purified approximately 1400 fold (80-90% purification) using mouse liver from A/J mice ($H-2^a$ haplotype). The large scale procedure developed here routinely provides 2-3 mg of heavy chain ($H-2K^k$ plus $H-2D^d$) from 1 kg of mouse liver. Partial amino acid sequencing has now been possible for the first time for H-2. (See Appellas' annual report for details.) Hopefully we shall now proceed to study the biological effects of the purified material. The material at the 500 fold purification level was not as active in inducing tolerance etc. as the material recovered after G-150 Sephadex fractionation.

2. H-2 antigens in a modified form or as derepressed antigens have been implicated indirectly by several investigators in host immune responses to tumors - specifically evidence points strongly to H antigens, in some form, to be tumor specific antigens (of the transplantation type). We are looking for such evidence in several systems:

a). Since alien (or inappropriate) H-2 specificities have been described in several methylcholanthrene-induced sarcomas in BALB/c mice, particularly by Parmiani and Invernizzi of Milan, we have studied a series of 7 MCA-induced neoplasms of BALB/c (from pedigreed lines to assure non-contamination). Through the use of in vivo immunogenicity assays - growth and the induction of immunity in various F_1 hybrids with BALB/c, we have found no evidence for inappropriate or alien H-2 determinants and in limited studies using serologic assays, no evidence was found for any H-2 determinants except those public and private specificities determined by $H-2D^d$ and $H-2K^d$ loci. Thus, all of these sarcomas do have individually distinct TSTA and at least no detectable modified or derepressed H-2 antigens appear to be functional as TSTA.

b). Studies continue concerning characterizing the set of alien specificities (H-2, 1, 5, 11, 23 and 25) representing the $H-2K^k$ haplotype on Parmiani's C-1 MCA-induced sarcoma. Two questions of major interest are, 1) does this molecule act as the tumor specific transplantation antigen (TSTA) of the tumor - as claimed on indirect evidence and 2) is the molecule the same or different from the normal $H-2K^k$ molecule (e.g. from C_3H cells)? The following pertinent facts are now known (See Rogers' annual report) comparing the normal and alien H-2 antigens from purified membranes of C-1 cells: 1) The molecular weight of the molecule bearing alien specificities is 50% greater (150,000 daltons) than the normal (100,000) in gel filtration; both are glycoproteins. 2) The normal antigen, as most H-2 heavy chains, binds β_2-microglobulin tightly whereas there is no binding or very loose binding with the alien antigens. 3) There is very little of the alien antigens on the cell (as much as 100X less) compared to normal H-2; this of course could reflect differences in binding, 4) Whereas normal H-2 antigens resist papain digestion, the alien antigens are very sensitive - and most may even be destroyed by papain. Although the specificities as detected serologically are all present on the C-1 molecule and appear similar to normal $H-2K^k$, the above results tend to indicate that the "alien" specificities and normal specificities are on different molecules. One additional biologic difference is reflected in the C-1 alien antigens' inability to evoke a cellular response - contrary to the normal antigens.

Work is progressing on the purification of the alien antigen to compare it with H-2 and TSTA and to determine in a more definitive way if this alien antigen is a tumor specific antigen.

II. Tumor Specific Antigens

1. We have returned now (with Appella and DuBois) to attempts at purification of the individually distinct TSTA on Meth A sarcoma cells. Previously with Natori we had achieved good isolation but with only minimal purification over the specific activity of the crude membrane (CM) preparation. Meth A has certain advantages over other systems: a) the neoplasm exists in ascites form, b) it has extremely strong TSTA, c) it has no detectable alien H-2 specificities and d) the in vivo passaged line has no infectious C type virus or virus fingerprints. In addition Dr. Old's group at Memorial-Sloan Kettering have developed a tumor-specific antibody against Meth A and hopefully a competition-binding assay can be developed to aid in assaying fractions during purification; we are cooperating with the SK group in this aspect of the study.

We have found in this new study that purified membranes solubilized with NP40 and chromatographed on AcA22 columns that extremely small amounts from Fraction III, subsequently fractionated on Lens culinaris lectin and wheat germ agglutinin columns (to completely remove H-2 and β_2-microglobulin, retained specific tumor rejection activity against Meth A; 95% tumor inhibition was achieved with as little as 1.0 μg protein. AcA 54 chromatography following lectin fractionation has resulted in a major peak (60,000 daltons) of TSTA activity. This material gave only 1 band on SDS gels. It is our feeling that purification will be greatly enhanced now beginning with the 60,000 dalton material from AcA 54 gel filtration and work is continuing with this material.

All biologic assays we have completed with our solubilized, partially purified material indicate activity similar to that observed using intact Meth A cells - thus the antigen is not degraded, specific immunogenicity is maintained, there was observed no "immune deviations" etc.

2. SV40 Tumor Antigens

a) SV40 Tumor Antigens as detected in the mouse and in the Syrian hamster.

Extensive cross-reactivity studies have been reported for the nuclear T antigen; antigenically related T-ag has been demonstrated in SV40 transformed cells from different species and those induced by the variant viruses recovered from man: DAR, BK and JC. We have previously reported cross-reactivities of TSTA of SV40-induced neoplasms of several species, man, rat, sheep, hamster, mouse etc. employing the mKSA system in BALB/c mice. Intact cells, plasma membranes and solubilized TSTA were equally effective. When, however, we employed the immunosensitive SV34 tumor of the hamster we found that whereas all SV40-, BK- and JC-induced neoplasms, membranes and solubilized ag immunized the mouse, only SV40 virus and SV34 intact calls (not membranes nor soluble antigen) were capable of immunizing the hamster. These results strongly suggest the existence of different species of SV40-encoded TSTA. We have developed a concept to explain these findings in terms of different species of TSTA in the mouse and hamster. (See Law, Takemoto, Rogers, Henriksen and Ting, Int. J. Cancer 1978, in press).

More recently and equally interesting are the findings of Takemoto and Law that neoplasms induced in the hamster by SV40, BK and JC viruses respectively have their own individually distinct tumor rejection antigens (TSTA) that do not cross-react. It has been reported however, that the T-antigens of these neoplasms are antigenically related.

b). We have found (with M. Rogers) that the TSTA from SV40-induced neoplasms of the mouse (mKSA) can be obtained simply by homogenization of dissociated cells and AcA chromatography without resort to solubilization with detergents. We have found that this soluble antigen retains all the biologic and immunogenic characteristics of the intact cell: immunization is achieved easily with a single dose of 10^6 cell equivalents, this immunization is specific for SV-40 induced neoplasms, the immunity provided by soluble antigen is stable and long lasting (150 days), there is no "overloading" with large doses of antigen. This soluble SV40 antigen is now being used in attempts to induce secondary cytolytic T lymphocytes in vitro (by Dr. M. Glaser). A more detailed understanding of molecular events involved in recognition in a syngeneic tumor-host system (as opposed to allogeneic systems) is therefore now possible.

3. Characteristics of TAA on FMR-MuLV-induced leukemias

A clearer picture of the tumor associated antigens (TAA) present on plasma membranes of leukemias induced by the FMR-MuLVs is now emerging as a result of studies (with Rogers) of the biologic activities of the different molecular entities obtained by solubilizing with Na-DOC and chromatographing using AcA 34 and Lens culinaris lectin affinity columns. The following findings are pertinent: a) Tumor rejection activity (TSTA) is solubilized and partially purified (based on yields of specific activity) and is found principally in high MW fractions (2, 3 and 4 of the AcA34 column), b) TSTA activity is clearly separated from H-2, gp70, p30 and p15E activity by gel filtration, c) The partially purified TSTA retains the specificity of the intact cells - providing tumor rejection against FMR leukemias but not against spontaneous and MCA-induced leukemias - nor against other neoplasms, d) A reasonable dose-response is observed at lower doses (from 1 μg to 25 μg) but interestingly doses of antigen (membranes as well as solubilized materials) > 25 μg do not provide rejection of RBL-5. The reasons for the induction of this "null" state is presently under investigation, e) A second antigen, unrelated to TSTA, and detected by in vitro cytotoxicity against RBL-5 employing syngeneic antisera, has been identified. This is a low MW entity (fraction 9 from the AcA34 column) and appears to be p15E. This antigen is unlike the TAA described by Fenyö and Klein present on Moloney virus-induced YAC cells and unlike the antigen on Moloney virus induced leukemia cells described by Ng; this latter appears to be related to viral structural proteins.

4. Immune Reactions of the Tumor Bearing Host

It is proposed during the coming year to examine in depth the factors responsible for the severe immune depression in hosts bearing progressively growing neoplasms. This work is a collaborative effort with Litton-Bionetics (Drs. Dean and Padarathsingh). By examining several different T-cell and antibody responses over the entire period of time from grafting to death, we have observed 3 different types of response - depending upon the tumor-host system used: a) during the progressive growth of mKSA, an SV40-induced sarcoma, a period of "eclipse" develops during which there is immune incompetence of a tumor specific nature while other T cell and B cell functions appear to be well preserved, b) during progressive growth of Meth A, an MCA-induced sarcoma, both tumor specific and generalized immune defects are the rule, c) while in BALB/c mice bearing Adj-PC5 plasma cell tumors tumor specific immunity is preserved in large tumor-bearing hosts while a striking immune deficiency appears in all other T-cell and B-cell reactivities. All of the above deficiencies are corrected by excision of the tumor.

Preliminary evidence has been obtained to implicate "suppressor" cells at least in the Adj-PC5 system. It is clear that if "suppressor" cells are involved, the mechanisms must be quite distinct for each host-tumor system. Thus, a major effort will be directed toward defining immune incompetence in these systems.

Significance for Cancer Research:

Characterization of tumor antigens of the rejection type is a necessary prerequisite for understanding the mechanisms of immune surveillance, tumor inhibition and facilitation. In addition the role of these membrane bound components in the actual initiation and maintenance of malignancy may be studied.

As a basis for any study of membrane bound antigens it is necessary to study the nature of histocompatibility antigens and their relationship to tumor antigens. Thus our emphasis is on parallel studies of H-2 antigens in order to provide a basis for understanding tumor antigens.

Objective 3; Approaches 4,5.

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Rogers, M. J., Law, L. W., Prat, M., Oroszlan, S. and Appella, E.: Separation of the tumor rejection antigen (TSTA) from the major structural proteins of an R-MuLV induced leukemia. Int. J. Cancer 21: 246-252, 1978.

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Padarathsingh, M. L., Dean, J. H., McCoy, J. L., Lewis, D. D., Northing, J. W., Natori, T. and Law, L. W.: Cell mediated immunity against particulate and solubilized tumor-associated antigens of murine plasmacytomas detected by macrophage migration inhibition assays. Int. J. Cancer 20: 624-631, 1977.

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Dean, J. H., Padarathsingh, M. L., McCoy, J. L., Jerrells, T., Lewis, D. D., Northing, J. W. and Law, L. W.: Evidence for tumor associated antigen-specific cell mediated immunity in plasmacytoma immune and tumor bearing mice. In Proceedings, 8th International Symposium on Comparative Leukemia Research, Amsterdam, (in press) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03229-09 LCBGY

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
I. Chemical structure of immunoglobulin molecules, allotypes and human histocompatibility antigens. II. Biochemical characterization of soluble murine H-2 antigens, tumor specific antigens and epoxide hydrase.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ettore Appella	Medical Officer (Res.)	LCBGY	NCI
OTHER:	Ole Henriksen	Sr. Staff Fellow	LCBGY	NCI
	John Inman	Biochemist	LI	NIAID
	Rose Mage	Immunochemist	LI	NIAID
	N. Tanigaki	Biochemist	Roswell Park	
	G. C. DuBois	Expert	LCBGY	NCI
	E. A. Robinson	Chemist	LCBGY	NCI

COOPERATING UNITS (if any)
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Laboratory of Immunology, NIAID

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.25	4.50	0.75

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chemical and Structural Studies of Antibodies and Histocompatibility Antigens. It is the purpose of this project to study the chemistry and structure of antibodies and histocompatibility antigens. Antibodies of different specificities are purified. The chemial sequence of both the light and heavy chains is obtained in order to gain some insight into the nature of antibody specificity and the generation of diversity. Isolation methods for histocompatibility antigens and tumor antigens have been worked out. The characterization of the chemical structure of these proteins is being approached by micro methods and the relationship of the amino acid sequence to immunoglobulin structure or other known structures is being analyzed. Epoxide hydrase plays a critical role in chemical-induced mutagenesis and carcinogenesis. Structural studies have been undertaken with the goal of identifying aminoacid residues which are essential for catalytic function.

Rabbit Immunoglobulins:

Comparison of the amino acid sequences of many rabbit light chains have revealed two segments of hypervariability which correspond to the first and third hypervariable regions found in the light chains of other species (residues 24-34 and 89-97). These regions are involved in forming the antigen binding site and are termed complementarity-determining regions. Elicited rabbit antibodies to type III and type VIII have markedly different hypervariable sequences and lengths.

In a partially inbred colony 6 light chains from elicited antibodies to streptococcal group A variant carbohydrate have been reported with identical sequences of the first hypervariable region. We have studied a light chain from a rabbit antibody to type III polysaccharide with a sequence strikingly similar to the light chain from anti-A variant antibodies and therefore belonging to the same subgroup as the previous light chains. More interesting was the finding that residue positions in the first hypervariable region were identical. The major differences between the two types of chains were found in the third hypervariable region, although they were identical in length. Several differences were also found outside the hypervariable regions, indicating that this single subgroup is represented by more than one germ-line gene. The question arises why light chains of anti-type III and anti-A antibodies of unrelated rabbits share the first hypervariable region and yet are very different in the third hypervariable region. One likely possibility is that the first hypervariable region is not participating in antigen binding, in either case; however, more work such as heterologous chain recombination and determination of the binding capacity for either antigens required to assess correctly the significance of our finding.

Mouse Immunoglobulins:

Comparison of the complete amino acid sequence of the shortened heavy (α) chain of mouse IgA47 to that of a human α chain revealed that the entire C_H3 domain of the 47A α chain had been deleted and that the hinge region differed both in length (seven residues shorter) and sequence. The sequence of a non-deleted mouse α chain (MOPC 511) has been undertaken in order to provide a more meaningful comparison to the deleted 47A chain. The data obtained thus far show that the hinge regions of the two mouse α chains are identical and that the C_H1 and C_H2 domains are probably identical except for a single interchange of threonine to alanine. Cys-135 of 47A which participates in the light-heavy chain band is also present in the 511 λ chain despite the fact that the chains of 511 are not covalently linked. It has been tentatively concluded that IgA 47A is a mutated allotype of IgA 511. The absence of C_H3 domain in 47A apparently induces a profound conformational change which not only presents the formation of a four-chain molecule, as in 511, but allows the formation of a light-heavy disulfide bond.

Histocompatibility and tumor specific transplantation antigens:

The heavy chains of papain-solubilized $H\text{-}2^a$ antigens ($H\text{-}2K^k$ plus $H\text{-}2D^d$) have been purified to homogeneity and chemically characterized. The molecules consist of 290 amino acid residues, contain two disulfide bridges and one free cysteine residue, and have homologus NH_2- and COOH-terminal sequences. The NH_2-terminal sequences of the H-2 chains closely resemble those of HLA. However, the solubilized mouse and human histocompatibility antigens differ in chain length, COOH-terminal sequence, and cysteine content. These observations suggest that papain cleaves membrane bound H-2 and HLA at different positions with respect to the COOH-terminus.

A serum component that appears to be shed H-2 antigen has been purified from serum of Swiss BALB/c mice strain. It represents the major β_2-microglobulin bound component in serum and contains in addition to β_2, another polypeptide of approximately 38,000 daltons. The larger polypeptide chain has an amino acid composition closely related to the large peptide of H-2 antigen solubilized by papain. It lacks alloantigenic specificities but shares with H-2 xenoantigenic determinants detected by a rabbit antiserum raised against the shed, H-2. This molecule could represent an H-2 which is negative for alloantigenic specificities but still retains the basic structure of H-2.

Individually distinct tumor rejection antigens (TSTAs) are associated with chemically induced neoplasms regardless of species. Efforts have been devoted to solubilize and purify these TSTAs, with retention of tumor rejecting activity. By use of NP40, we have succeeded in solubilizing in good yield the TSTA of Meth A, a methylcholantrene induced sarcoma of BALB/c origin. This antigen does not appear to be degraded, since a single molecular species of approximately 60,000 has been isolated. H-2 antigen, β_2-microglobulin and viral structural proteins (gp70, p30) were separated from TSTA. An antiserum was raised in rabbits against a partially purified antigen preparation. When absorbed in vivo, this antiserum retained specificity for a Meth A surface antigen. A radioimmunoassay was established and by inhibiting the binding of partially purified radiolabeled antigen to the absorbed antiserum, it was shown that the surface antigen could be purified, about fifty fold. The TSTA activity was retained during each stage of purification. At this stage, however, it is not known if the tumor specific surface antigen and the TSTA are part of the same molecule or are different molecules of the same molecular weight and very close ionic composition. Final purification we hope will fully answer such a question, as well as its relationship to H-2 antigen or other antigens which might be defined on the Meth A cells.

Epoxide Hydrase:

Efforts have been made to determine if epoxide hydrase exists as a single protein chain or two or more isozymes. Carboxypeptidase Y digestion of the purified enzyme reveals the presence of only one carboxyl terminal amino acid (leucine) per mole of protein. The carboxyl terminal sequence deduced from this study is - VAL-GLU-ALA-LEU. Amino terminal analysis by the dansyl method failed to show any N-terminal. However, by automatic Edman degradation, lysine was tentatively identified as the N-terminus and the sequence was determined for an additional 18 residues. The N-terminal sequence of the first 19 residues is marked by the presence of a high number of hydrophobic amino acids. This hydrophobic region may serve as a site of attachment to the membrane surface of the endoplasmic reticulum. Thus, the data accumulated so far support the premise that there is a single form of epoxide hydrase which possesses very broad substrate specificity.

In order to identify a partial amino acid sequence containing the N-alkylated histidine derivative, peptide mapping studies have been undertaken. A 9mg sample of the modified epoxide hydrase (1.1 moles of ^{14}C-Br-4-NAP/mole of protein) was succinylated with succinic anhydride to mask the lysyl residues. The protein was reduced and carboxymethylated, and digested with trypsin. Gel filtration chromatography or Sephadex G-75 revealed the presence of the protein which eluted with the void volume. The presence of two ^{14}C-labelled peptides is probably a cleavage product of the partially digested larger peptide. Experiments are currently in progress to purify and sequence both of these peptides.

Studies on Alien H-2 Specificities:

Drs. Prat and Rogers have examined the probability of existence in a chemically induced leukemia of SJL/J mice (H-2^s haplotype) of alien H-2 specificities detected by complement-mediated lysis. This anomalous reactivity was found to be due to previously undetected public specificities that were also found in normal cells of the H-2^s haplotype.

Publications:

Henriksen, O., Law, L. W. and Appella, E.: Quantitative *in vivo* characterization of soluble SV40 tumor specific transplantation antigen (TSTA) of the mouse. *J. Nat. Cancer Inst.* 58: 1785-1788, 1978.

Rogers, M. J., Law, L. W., Appella, E., Oroszlan, S. and Ting, C. C.: The role of the major viral structural proteins gp70 and p30 in the immune reponse to murine leukemia induced by Friend or Rauscher virus. *J. Nat. Cancer Inst.* 20: 303-308, 1978.

Rogers, M. J., Law, L. W., Prat, M., Oroszlan, S. and Appella, E.: Separation of the tumor rejection antigen (TSTA) from the major viral structural proteins associated with the membrane of an R-MuLV-induced leukemia. *Int. J. Cancer* 21: 246-252, 1978.

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Inman, J. K., DuBois, G. C. and Appella, E.: Synthesis of macroporous polystyrene derivatives for supports in sequential degradations of peptides, in solid phase methods in protein sequence analysis. In Proceedings of the Second International Conference on Solid Phase Methods. North-Holland, Amsterdam, Montpellier, France, 1977. A. Previero and M. A. Coletti-Previero, Eds.

Appella, E., Inman, J. K. and DuBois, G. C.: Solid phase degradations on macroporous polystyrene derivative with identification of thiazolinones as PTC amino acid methylamides, in solid phase methods in protein sequence analysis. In Proceedings of the Second International Conference on Solid Methods. North-Holland, Amsterdam, Montpellier, France, 1977. A. Previero and M. A. Coletti-Previero, Eds.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03255 -03 LCBGY

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immune Response to Tumor Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. C. Ting	Medical Officer	LCBGY NCI
OTHER:	L. W. Law	Chief, LCB	LCBGY NCI
	M. J. Rogers	Senior Staff Fellow	LCBGY NCI
	T. Igarashi	Guest Worker	LCBGY NCI
	T. Natori	Expert Consultant	LCBGY NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS: 3.50	PROFESSIONAL: 2.50	OTHER: 1.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Studies of the mechanism for the induction of in vivo tumor immunity: By employing both in vitro and in vivo techniques, the distribution and homing of cytotoxic effector cells and precursor cells could be examined, and the interaction of various cellular and humoral elements in the immune response could be studied.

2. Humoral and cellular regulation of cell-mediated immunity: Various humoral and cellular factors were identified to be able to suppress or augment the cell-mediated immunity. Some of them could reverse the immunosuppressive effect caused by tumor cells in vivo.

3. Immunosuppression in cancer patients: An immunosuppressive factor(s) was demonstrated in cancer patients' sera that could selectively suppress the T cell functions.

Project Description:

Objectives: The objectives of this project are to study: 1) The mechanisms for the induction of *in vivo* tumor immunity; 2) Humoral regulation of cell-mediated immune response; 3) Reversal of immunosuppression; and 4) Immunosuppression in cancer patients.

Methods Employed: Tumor cell transformed by oncogenic viruses, chemicals or unknown agents are kept in the tissue cultures or as transplanted tumors in mice. The ^{125}IUdR release assay was used to measure the cell-mediated cytotoxic responses *in vitro*, and the adoptive transfer experiment was used to measure the cell-mediated cytotoxic responses *in vivo*. The *in vivo* tumor immunity was tested by transplantation experiments.

Major Findings:

I. Mechanisms for the induction of *in vivo* tumor immunity: The adoptive transfer experiment which has been developed to study a series of reactions that may be involved in the *in vivo* immune response gave excellent correlation to the *in vivo* tumor immunity in all aspects that we have studied so far. We can study the distribution pattern and homing of cytotoxic effector cells and memory cells in the adoptively transferred recipients. This technique also allows us to examine the roles of various cellular and humoral elements in the induction of an efficient immune response.

II. Humoral regulation of cell-mediated cytotoxic response. We have found that a T cell helper factor was present in the normal serum of different species. This factor(s) could augment the T cell-mediated response of allogeneic mixed lymphocyte cell culture reactions and syngeneic mixed lymphocyte tumor cell culture reactions. The mechanism for the augmentation appeared to be mediated by the generation of helper cells by this humoral factor. Thus, humoral and cellular regulation of immune response appears to be interrelated.

III. Reversal of immunosuppression. In many cases the lack of detectable immune response was due to the presence of suppressor cells or immunosuppressive factors. In studying the immune response to FBL-3, a Friend virus-induced leukemia in C57BL/6 mice in syngeneic and allogeneic system, we have found that FBL-3 cells were immunosuppressive *in vitro*; however, its immunosuppressive effect could be overridden by the host *in vivo*. Thus, the ability of the hosts to reject the tumor could be partly attributed to its ability to override the immunosuppressive effect of tumor cells. Both cellular and humoral elements were found to be involved in the reversal of immunosuppression.

IV. Immunosuppression in cancer patients. We have tested sera and tissue fluids from cancer patients and patients with acute and chronic inflammatory diseases, and normal individuals. It was found that an immunosuppressive factor was present in most cancer patients and patients with chronic and acute inflammatory diseases, but not in normal individuals. This factor could selectively suppress the T cell response with preservation of B cell response.

Significance for Biomedical Research and the Programs of the National Cancer Institute:

Through the use of various in vitro and in vivo techniques, we are able to study the mechanisms for the induction of in vivo tumor immunity, and the interactions between different cellular and humoral elements involved in various phases of immune response. We have found that although T cells were mainly responsible for the in vivo tumor immunity, other cellular and humoral elements also played important roles in the generation of an efficient immune response. The study of these fundamental problems should help us to find ways and means to monitor the immune response that will be beneficial to the hosts.

Proposed Course:

I. To study the mechanisms for the induction of in vivo tumor immunity. To continue the search of various cellular and humoral elements that are involved in the immune response reactions.

II. Reversal of immunosuppression: Attempts will be made to find efficient ways to reverse the immunosuppression caused by tumor cells.

III. Characterization of immunoregulatory cells and immunoregulatory factors and the mechanisms for their actions.

Publications:

Ting, C. C.: Studies of the mechanisms for the induction of in vivo tumor immunity. II. Distribution and homing of cytotoxic effector and precursor cells. J. Natl. Cancer Inst. 60: 437-444, 1978.

Ting, C. C., Sanford, K. K., and Price, F. M.: Expression of fetal antigens in fetal and adult cells during long-term culture. In Vitro 14: 207-211, 1978.

Ting, C. C., and Rule, A. H.: Oncofetal antigen (workshop). In Progress in Immunology III. Australian Academy of Science, Canberra City. T. E. Mandel, C. Cheers, C. S. Hosking, I. F. C. McKenzie, and G. J. V. Nossal (Eds) 1977.

Ting, C. C., Rodrigues, D., Hersh, E., and Hirschl, S.: Selective suppression of T cell-mediated immunity by cancer patients sera. Proc. XII International Congress of Immunology (in press) 1978.

Rogers, M. J., Law, L. W., Appella, E., Oroszlan, S., and Ting, C. C.: Solubilized TSTA and the major viral structural protein, gp70 and p30, in the immune response to murine leukemia induced by Friend and Rauscher virus. Int. J. Cancer 20: 303-308, 1977.

Terry, W. D., and Ting, C. C.: Immunology. In Cancer in China. H. Kaplan and P. Tsuchitani (Eds.) Alan R. Liss, Inc. (in press) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 04833-09 LCBGY

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biological Studies of Various Normal and Virus-Infected Malignant Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. A. Wivel	Head, Ultrastructural Biol.	LCBGY	NCI
	S. S. Yang	Chemist	LCBGY	NCI

COOPERATING UNITS (if any)

R. Gantt	LRTV NCI
P. Pitha	Johns Hopkins University School of Medicine

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Ultrastructural Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the primary purpose of this project to study some of the pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches which range from the microscopic to the molecular level. Particular emphasis is given to those systems in which murine RNA tumor viruses or their precursors may be the transforming agent. A variety of mouse model systems are used, including plasma cell tumors, mammary tumors, and neuroblastomas. Current projects include: 1) characterization of intracisternal A particles (IAP) with particular emphasis on homology to known RNA tumor viruses, molecular events associated with replication, conservation and divergence of IAP genes, and factors controlling the expression of IAP genes; 2) effects of interferon on the assembly and maturation of murine oncornaviruses with special emphasis on the study of mechanisms whereby whole virions are rendered noninfectious.

PHS-6040
(Rev. 10-76)

Major Findings:

1. Intracisternal A particles. A collaborative study with Dr. Raymond Gantt has yielded additional information about certain events which occur during the formation of intracisternal A particles (IAP). Since it had previously been shown that several murine and avian retroviruses had an associated N^2-guanine RNA methylase, and since IAP exhibit several characteristics of retroviruses, it seemed pertinent to determine if such an enzyme was associated with IAP. The results of this investigation indicated that six methylases were associated with IAP, a cytidine and a uridine methylase, two N^2-guanine monomethylases, an N^2, N^2-dimethyl-guanine methylase, and an adenine methylase. Using E. Coli phenylalanine tRNA as an acceptor RNA, these enzymes were shown to be indistinguishable from cellular enzymes. However, it is of significance that the specific activity of these enzymes increased about two fold when IAP cores were prepared by stripping the outer envelopes with 1% Triton X-100; this result strongly suggests that the methylases are present inside the particles and not just absorbed to the surface. It seems probable that these cellular enzymes are packaged within IAP during the process of synthesis. The function of such RNA methylases is not known, but it is of interest that an analogous situation exists in the case of Rous sarcoma virus and avian myeloblastosis virus wherein cellular tryptophan tRNA is intimately associated with the 70S RNA and can serve as an effective in vitro primer of DNA synthesis.

There is abundant evidence that intracisternal A particles are not released from cells. Thus it is not surprising that a variety of cell cocultivation experiments have failed to demonstrate successful horizontal transmission. This failure of release can be abrogated in at least two ways. One, IAP-containing cells can be fused to particle-negative cells, in the presence of inactivated Sendai virus, and IAP are expressed as a dominant genetic trait in the resulting hybrids. Two, cytoplasts (enucleated cells) can be used as carriers of the IAP phenotype and when fused to particle-negative whole cells, the resulting cybrids contain IAP which are not segregated over long term passage. Therefore a form of horizontal transmission has been achieved. We have recently completed studies which more precisely define the nature of the infectivity associated with IAP. Using the endogenous reverse transcriptase associated with IAP, it has been possible to generate a cDNA probe and determine the approximate number of gene copies in the parent cells, the hybrids and the cybrids used in the fusion experiments. It was initially established that there was homology between cDNA probes derived from IAP purified from either plasma cell tumors or mammary tumors. Mammary tumor cells were used as IAP donors and two particle-negative lines, 3T3-4EF (NIH Swiss) and II6C (feral) were used as recipient cells. The MT cells contained 20-24 copies of IAP-specific DNA sequences per haploid genome and the hybrid cells, which contain the MT nucleus, had about the same or a slightly greater number of IAP sequences than the parent MT cells. The 3T3-4EF recipient cells had 1 or less IAP copies per haploid genome and the II6C cells had about 4 copies per haploid genome. Therefore two mechanisms can be invoked to explain the observed results. In the case of the MT-II6C cybrids, the presence of IAP suggest that a derepressor in the MT cytoplasts may have switched on the preexisting IAP genes in the II6C cells. In the case of the MT-3T3-4EF cybrids, which contained 15-20 copies per haploid genome, the presence of IAP may well reflect the possibility that the IAP genome was transferred from the MT cytoplasts to the 3T3-4EF DNA by reverse transcription, with subsequent integration and production of particles. Such a sequence of events suggests an analogy to the replication of retroviruses.

2. Interferon treatment of murine oncornaviruses. In a continuing collaborative effort with Dr. Paula Pitha, it has been possible to elucidate some of the effects of interferon on chronic in vitro MuLV infection. The effect of interferon on many different types of viruses is a fairly specific one; there is an impairment in the translation of viral mRNA with resulting defects in virion polypeptide synthesis. In the case of MuLV, the interferon-induced inhibition occurs following the synthesis of the majority of viral proteins. It is likely that such inhibition is mediated through changes in cellular membranes with subsequent defects in virus assembly and maturation. Our recent studies have established that the stage of the block in virus assembly is dependent on the host cell used to replicate the virus. In AKR-L1 cells there is an arrest at the budding stage and in SC-1 cells the arrest is later in assembly with a marked accumulation of whole virions at the plasma membrane of the cell. The virus particles in SC-1 cells are morphologically identical, whether produced in the presence or absence of interferon. However a significant percentage of both cell-associated and released virus particles subjected to interferon treatment were non-infectious. This decrease in infectivity may not be mediated through the antiviral effect of interferon. The drop in infectivity of MuLV preceded any reduction in the yield of virus particles; it has been possible to demonstrate that infectivity can be reduced 10 fold when the amount of virus is reduced only 3 fold. Infectivity was reduced in 3-6 hours after addition of interferon, while the antiviral state, as measured by the inhibition of vesicular stomatitis virus (VSV) replication, was detected only after 8 hours of interferon treatment. The observed time difference required for the inhibition of MuLV and VSV is not caused by the greater sensitivity of MuLV to interferon. When measured in the first replicative cycle, MuLV is 3-fold less sensitive to interferon than VSV.

In order to better understand the basis for the loss of virus infectivity, we have studied the effects of interferon on the synthesis of the major virion polypeptides of MuLV. SDS polyacrylamide gel profiles of infectious and noninfectious virus were compared. Noninfectious virus had an additional band in the 90 K dalton region which was absent in the controls; this 90 K protein could be labeled with ^{3}H-glucosamine but not with ^{3}H-fucose. To determine whether this 90 K glycoprotein was related to the precursor to the major envelope protein, gp 71, ^{3}H-glucosamine labeled virions were precipitated with antiserum to gp 71. In the untreated control virus, only the gp 71 was precipitated, but in the interferon treated virus both the 90 K glycoprotein and gp 71 were precipitated; since gp 90 is fucose deficient and since it shares an antigenic determinant with gp 71, it is a candidate as the precursor of the major envelope glycoprotein. These results raise the distinct possibility that there is an impairment in the cleavage of gp 90 to gp 71 with a resulting loss in infectivity. Current efforts are directed toward a more complete analysis of this phenomenon.

Publications:

Potter, M., Premkumar, E., and Wivel, N. A.: Immunoglobulin production by lymphosarcomas induced by Abelson virus in mice. In Sanford, K. K. (Ed.): Third Decennial Review Conference on Cell, Tissue and Organ Culture. NCI Monograph Series. 48:396-408, 1978.

Pitha, P. M., Maldarelli, F., Hattman, T., and Wivel, N. A.: "Env" gene precursors in Rauscher MuLV produced in the presence of interferon. Nature, In Press.

Pitha, P. M., Wivel, N. A., Fernie, B. F., and Harper, H. P.: Effect of interferon on MuLV infection. IV. Formation of noninfectious virus in chronically infected cells. J. Gen. Virology, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 04834-02 LCBGY

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Viral Gene Expression in Normal Tissues and in Virus- or Chemically-induced Neoplasms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Ultrastructural Biology

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.30	.30	1.00

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major thrust of this study is to elucidate the basis of neoplastic transformation of normal tissues. Two experimental model systems were used in this project: (1) Intracisternal A particle (IAP) containing neoplasms, a BALB/c mammary tumor cell line, its hybrid and cybrid clones, a plasma cell tumor (MOPC 104E), and feral mouse cell lines of both Mus musculus and Mus cervicolor; and (2) a rat hepatoma induced by the chemical carcinogen, N,N-2,7-fluorenylene bis-2,2,2-trifluoroacetamide. Multidisciplinary approaches were required in this investigation. They involve tissue culture, biochemical and biophysical analyses, cytogenetics, immunology and tumor biology. Since we have completed extensive characterizations morphologically, biologically, biochemically and immunologically of both these retraviruses, our current emphasis of research efforts focuses on: (1) integrations of horizontally transferred viral genome and its localization in the host cell; (2) regulation of viral gene expressions; (3) integration site(s) for exogenous viral DNA sequences; (4) viral gene conservation during the evolution of the Mus genus; and (5) resolution of the GAG and ENV gene products (gs antigens and envelope antigen).

Major Findings:

(1) Integrations of horizontally transferred viral genome and its localization in the host cell and (2) regulation of viral gene expressions.

Intracisternal A particles (IAP), Retroviridae, are distinctive in that they are confined to cytoplasmic cisternae of various tumor cells (myeloma, mammary tumor, neuroblastoma, rhabdomyosarcoma, and yolk sac carcinoma), embryonic cells, and rarely in normal adult cells. When isolated and purified from murine tumors, they have never been shown to be infective. We have in the past cultivated mouse cell lines, which were negative for the IAP phenotype, with whole cells, subcellular fractions and purified IAP without successful stimulation of IAP production in these negative recipient cells. The lack of infectivity of isolated, purified IAP may reflect impaired entry and uncoating within the host cell; additionally, the potential infective capacity of IAP is significantly compromised by an isolation procedure which requires detergent treatment. Until now, horizontal transfer of IAP could only be demonstrated by fusion of IAP-positive cytoplasts, derived from mammary tumor cells (MT), with IAP-negative recipient cells. In this model for horizontal transfer of the IAP genome, we have used as the IAP donor, a continuous cell line established in this laboratory and derived from a BALB/c mammary tumor. As recipients, two phenotypically negative cell lines were used, 3T3-4EF cells (NIH Swiss) and the II6C clone of SC-1 cells (feral). Mammary tumor whole cells or cytoplasts (enucleated cells) were fused to these lines. The resulting hybrids and cybrids have previously been well characterized. Using a radioactive cDNA probe synthesized by the reverse transcriptase reaction of MT IAP for DNA-DNA hybridizations, we have approximated the number of copies of IAP-specific DNA sequences in the parent cells and their related hybrids and cybrids. The purpose of this study is to define more precisely the mechanisms of IAP phenotypic transfer and in particular to determine whether IAP-specific DNA sequences already exist in these recipient cells or whether such DNA sequences are integrated de novo as a result of the fusion process.

Based on the copy numbers of IAP-specific DNA sequences observed in the various parent cells, hybrids, and cybrids, and the thermal stability characteristics of the DNA duplexes, two mechanisms were proposed for IAP expression in the cybrid cells which were formed by the fusion of IAP-positive cytoplasts with IAP-negative cells. First, in the case of a genotypically negative recipient cell, 3T3-4EF, fused with the IAP donor MT cytoplast (cybrid clone J18) the copy numbers of IAP-specific DNA sequences increased from 1 to 10. The horizontal transfer is probably mediated by a reverse transcription of the IAP genome, followed by subsequent de novo integration and transcription of the DNA sequences, thus accounting for the continuous replication of IAP in the cybrid cell. By cell fractionation, we separately isolated and purified the cytoplasmic DNA from the nuclear DNA derived from five grams of J18 cybrid cells. We found that the de novo integrated IAP-specific DNA sequences all resided in the nuclear DNA fraction, implicating that such sequences have been permanently integrated into the cell genome.

Second, in the case of the D4 cybrid cells, in which II6C, the phenotypically IAP-negative feral mouse cell, already carries the IAP genome, IAP transfer by cybridization with the MT cytoplast did not increase the number of IAP-specific sequences. The IAP expression in the D4 cybrid cells may be explained by the derepression of a regulator gene controlling the expression of IAP in II6C, due to a factor transferred from the MT cytoplast. It seems therefore the expression of IAP also comes under the control of a regulator gene.

(3) Integration site(s) for exogenous viral DNA sequences.

Another finding of this study concerns the number of integration sites for IAP in the cell DNA. The data derived from these particular cell lines suggest that there is an upper limit to the number of IAP-specific sequences that can be incorporated into the cellular genome. There are about 10 copies of IAP-specific DNA sequences per haploid genome of MT cells, the MT-3T3-4EF hybrids show an additive effect with about 20 copies, and the MT cytoplast x3T34EF (J18) cybrids contain about 10 copies. These results are consistent with the hypothesis that both MT and 3T3-4EF cells can incorporate about 10 copies of IAP-specific sequences per haploid genome. A similar limit is observed in the case of II6C cells which contain 4 sequence copies per haploid genome both before fusion and after being fused as cybrids. While the derepression, a pre-existing genome would explain the results seen in the MT-II6C cybrids, it is also possible that other related interactions could occur as well. The pre-existing copies of IAP DNA sequences could have effectively blocked all the available integration sites or the cDNA generated by the IAP donated from the MT cytoplasts could have replaced the preexisting copies at the time of integration. Evaluation of these possibilities would be difficult in this system. However, there is a precedent for the limit in copy numbers that can be integrated into cell DNA in exogenous infection of mouse cells with AKR MuLV.

(4) Viral gene conservation during the evolution of the Mus genus.

A survey of the IAP-specific DNA sequences in laboratory strains of mice as compared to feral mouse cells in *Mus musculus* and *Mus cervicolor* indicates that the IAP genome is highly conserved. A T_m (thermal denaturation) variance of 4° plus the increase in copy number of IAP-specific DNA sequences suggests that there has been a relatively recent increase in the rate of divergence of such sequences in the laboratory strains of *Mus* bearing different types of tumors. Perhaps this is in some way related to the fact that the maximal expression of IAP occurs in tumors. The dichotomy of feral mouse versus inbred strains of laboratory mouse in which this difference in the evolving rates of a viral gene became apparent, has previously been documented in the MMTV-infected laboratory strains as compared to the feral mice of various species of *Mus*.

(5) Resolution of the GAG and ENV gene products (gs antigens and envelope antigen).

In an ongoing project the GAG and ENV gene products of two rat viral complexes were exhaustively analyzed by biochemical and radioimmunological techniques. One of the rat viral complex, KiMSV(RHHV) was isolated in this laboratory. By equillibrium polyacrylamide gel electrophoresis and fluorography, we have found that both viruses, when their proteins were chronically labelled with either ^{35}S-methionine or ^{3}H-amino acid mixture and isolated by immune precipitation with anti-RaLV serum, possess four proteins at variant amounts: these are 110-120,000 mol. wt. (P110-120), 70,000 mol. wt. (P70), 30,000 mol. wt. (P30), and 7-10,000 mol. wt. (P7-10). Among these proteins, the P70 and P110-120 are both glycosylated since radioactive glucosamine or galactosamine were both incorporated into the same viral protein peaks. Other glycosylated proteins associated with the virus are the 180,000 mol. wt., gp180, the 85,000 mol. wt., gp85, and the 40-45,000 mol. wt., gp40-45. Some of these large mol. wt. proteins or glycoproteins, gp180, P110-120 and gp85, were of cellular origin that were precipitated by the anti-RaLV serum during immune precipitation. That they may be precursors of the low mol. wt. structural proteins or glycoproteins have been investigated by pulse-chase experiments. The nature of the small mol. wt. P7-10 is not yet known.

Relationship of Research to National Cancer Plan:

Project Area - 10

Approach Elements - 2

Approach - 4

Publications:

Yang, S. S., Malech, H. M., Wu, R. S. and Woronow, D.: Properties of a transforming virus, KiMSV(RHHV) isolated from a co-culture of rat HTC-H1 cells with KNRK cells. J. Gen. Virol. 38: Part II, 209-225, 1978.

Yang, S. S. and Ting, R. C.: A comparative study of two viral complexes: MSV(RaLV) and KiMSV(RHHV). In Advances in Comparative Leukemia Research. Elsevier/Amsterdam, North-Holland, Biomedical Press, pp. 264-266. P. Bentvelzen et al. (Ed.) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05550-09 LCBGY

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Role of Retroviruses in Immunogenesis and Oncogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.00	1.50

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of the present project is to investigate the role of retroviruses in the etiology of reticulum cell as well as other lymphoid cell neoplasia, and to study the host responses, and functional markers and surface antigens of tumor cells.

The topics of current interest are: 1) Characterization of xenotropic retroviruses isolated from SJL/J strain of mouse which exhibit a high incidence of spontaneous reticulum cell neoplasm (RCN); and investigation of the role played by these viruses. 2) Isolation and characterization of a new dual tropic retrovirus from Abelson virus-induced lymphoma in BALB/c mice. 3) Natural killer cell activity exhibited by transplantable RCN of SJL/J mouse. 4) Tumor specific transplantation antigen(s) of RBL-3 (Rauscher virus-nonproducer) and RBL-5 (Rauscher virus-producer) cells. 5) Production of anti-viral and anti-FOCMA (Feline Oncovirus-associated cell membrane antigen) antibodies in guinea pigs.

PHS-6040
(Rev. 10-76)

Project Description:

I. Since previous experiments with an ecotropic virus (designated Dl-MuLV) isolated from SJL/J mice did not give conclusive evidence for its direct role in the etiology of RCN, the possibility of an involvement of xenotropic virus in this system was examined. Studies in AKR mouse indicated that an increased activity of ecotropic virus which by itself is not leukemogenic may lead to activation of xenotropic virus and manifestation of leukemia, with concomitant production of dual tropic, recombinant viruses. Although no such dual tropic virus has been isolated from SJL/J mice so far, the presence of xenotropic virus in the normal SJL/J mouse embryo and RCN (designated SJL/J-MEF-X-MuLV and SJL/J-RCN-X-MuLV respectively) suggests that the above mechanism of oncogenesis may be operative in SJL/J system. Toward this end, the xenotropic viruses isolated in this laboratory were first characterized by the following criteria.

a). Host range: By measuring at weekly intervals for 4 weeks the reverse transcriptase activity of virus pellets obtained from the culture fluid of infected cells, the following cells were found to be permissive for replication of both SJL/J-MEF-X-MuLV and SJL/J-RCN-X-MuLV: Human embryonic fibroblasts, human skin fibroblasts, a human osteosarcoma cell line, and a human conjunctiva cell line (WK-D); a mink lung cell line (Mv 1 Lu); a rabbit cornea cell line (SIRC); feline embryo fibroblasts; rat embryo fibroblasts and NRK (normal rat kidney) cell line. The following cells did not support the replication of these viruses. NIH-Swiss, BALB/c and C57BL/Ka mouse embryo cells; a wild mouse embryo cell line (SC-1); hamster embryo cells and BHK-21 cell line; a monkey kidney cell line (LLC-MK2); chick embryo cells. These results were essentially similar to those obtained with two other xenotropic viruses, AT124 virus isolated from NIH-Swiss mouse, and NZB virus isolated from NZB mouse embryos.

b). Template and ion preference in reverse transcriptase assays: Like other type-C viruses, these xenotropic viruses exhibited a preference for Mn^{2+} ion over Mg^{2+} ion when oligo (dT).poly (rA) was used as template. Oligo (dA).poly(rU) was less efficient (1/10) than oligo (dT).poly (rA) in the presence of Mn^{2+} for SJL/J-X-MuLV's, and AT124 virus, but these two templates were equally efficient for NZB-X-MuLV. In the presence of Mg^{2+}, however, oligo (dT).poly (rA) was inactive for all of these viruses tested, but oligo (dA).poly (rU) were moderately active for SJL/J-X-MuLV's and AT124, and much more active for NZB-X-MuLV. Therefore, SJL/J-X-MuLV's behaved more like AT124 than NZB-X-MuLV in these respects.

c). Viral interference patterns: SIRC cells preinfected with SJL/J-MEF-X-MuLV, SJL/J-RCN-X-MuLV, AT124, or NZB-X-MuLV were challenged with respective MSV pseudotypes and the resulting infectious centers (cells producing MSV pseudotypes) were assayed by plating the Mitomycin-C-treated SIRC cells on NRK cells. Such a two-step test was chosen because, although SIRC cells were more sensitive than NRK, the transformed morphology of SIRC cells made it difficult to assess the number of transformed MSV foci. The results indicated that there were reciprocal cross-interference between these viruses, suggesting that these viruses belong to the same class of X-MuLV's.

d). Virus neutralization tests: Rabbit antisera against these X-MuLV's were prepared and tested against MSV pseudotypes of these X-MuLV's for inhibition of MSV focus formation in NRK cells. In general, there were cross-neutralizations among these viruses, indicating their close antigenic relationships within the same class of X-MuLV's. However, there was a slight difference between AT124 and NZB-X-MuLV, and the SJL/J-X-MuLV's behaved more like AT124 than NZB-X-MuLV in the neutralization test. Fischer rat antiserum against ecotropic viruses, D1-MuLV (from SJL/J mouse), Moloney-MuLV, and AKR-MuLV were also tested along with the rabbit anti-X-MuLV sera in cross-neutralization tests. It was found that there was a certain degree of sharing of envelope antigens between SJL/J-X-MuLV's and D1-MuLV indicating the possibility of phenotypic mixing or recombinational events occurring in the SJL/J mice.

e). Further tests for comparisons of p12 antigenic differences among these viruses and BALB:virus-2 by competitive radioimmunoassay are in progress. These tests would indicate whether SJL/J-X-MuLV's are more related to one subclass of X-MuLV than to another, and help to elucidate the evolutional origin of these viruses. Further work performed to determine the frequency of isolation of X-MuLV's from normal spleens of SJL/J mice at different ages, i.e. prenatal, 3 weeks, 6 weeks, 3 months, 6 months, 9 months, and 12 months. The preliminary results indicated that there were more X-MuLV's at 6 and 12 months of age than at other ages, suggesting that the X-MuLV's are associated with higher frequency of spontaneous RCN occurring in the older age group.

II. Abelson virus was originally derived from a stock of Moloney-MuLV, but unlike the latter, induced non-thymic lymphoma in adult mice with a relatively short (3-4 weeks) latent period. The virus preparation appears to consist of a mixture of Moloney-MuLV (as a helper) and a defective transforming (leukemogenic) virus (Abelson-MuLV), since the latter can be rescued from a non-producer NIH/3T3 cell line (originally transformed by A-MuLV) by superinfection with helper viruses. The mechanism by which the leuc gene of A-MuLV induces lymphoma is of special interest.

a). Recently we have isolated a xenotropic virus and a dual tropic virus from an Abelson-virus induced lymphoma in BALB/c mice. The *in vitro* cultured tumor constantly produced ecotropic virus (NB-tropic, M-MuLV) in high titers (10^{5-6}), and a xenotropic and a dual tropic virus in low titers (10^{1-3}). By passage of the culture supernatants into feline and mink cells, and by repeated limiting dilution procedures to purify the virus clones and to rule out the phenotypic mixing, we finally succeeded in isolating a xenotropic virus and a dual tropic virus (designated CNBX). The latter showed an XC positive reaction, NB-tropism in mouse embryo cells, and a xenotropism as shown by its ability to replicate in heterologous cells such as human, mink, rabbit, cat, horse, rat, monkey, dog, chicken and duck cells. The dual tropic virus (CNBX) is being characterized by virological and immunological procedures to distinguish it from other dual tropic viruses such as HIX (P. J. Fischinger *et al.*) and MCF (J. W. Hartley *et al.*), and from wild mouse amphotropic virus (J. W. Hartley and W. P. Rowe). These viruses differed from each other in their ability to act as helpers for rescuing MSV from non-producer cells of cat, hamster, rat, human, and mouse origin, either by direct superinfection or by co-cultivation procedures. CNBX is distinguished from MCF virus and amphotropic virus (both are N-tropic) by its NB-tropism (ability to replicate in *Fv-1*^{bb} and *Fv-1*^{nb} mouse embryo cells), and is distinguished from HIX virus (which is also NB-tropic) by its ability to replicate considerably in hamster cells. Viral interference patterns suggested that CNBX is different from the amphotropic virus but is related to the group of dual tropic viruses, HIX and MCF. Further work is still in progress to characterize these dual tropic viruses by immunological methods and molecular hybridizations.

b). *In vitro* transformation of mouse lymphoid cells by A-MuLV: A-MuLV can transform mouse fetal liver cells into neoplastic lymphoid cells under *in vitro* cultivation conditions (N. Rosenberg *et al*.). We have confirmed this and further extended this observation to *in vitro* transformation of spleen cell suspension from adult mice. These transformed cells required the presence of 2-mercaptoethanol ($5X10^{-5}$M)in the medium for up to at least 7 passages *in vitro*. These transformed cells continuously grew in the medium while normal uninfected cells failed to grow indefinitely. Inoculation of syngeneic mice with the transformed cells resulted in typical Abelson tumors. Preliminary characterization of these transformed cells indicated the absence of Thy 1.2 antigen and the presence of surface immunoglobulin on the cell membranes suggesting their B-cell origin. The presence of transformed null cells is still being investigated. The availability of a rapid procedure for transformation of lymphoid cells provides an opportunity to test the behavior of *leuc* gene in xenotropic or dual tropic viruses mentioned above. Other leukemia viruses in our stock are also being screened for this ability. The functional markers and membrane antigens of transformed cell clones are also being investigated.

III. The SJL/J strain of mouse, which manifests a high incidence of spontaneous reticulum cell neoplasm (RCN) has B- and T-cell defects, and transplantable RCN of SJL/J origin exhibited a powerful cytotoxic activity against certain allogeneic tumor cells which was similar in many aspects to that of natural killer cells. The amounts of I^{125} released into the culture supernatant after 20 hr culture from [I^{125}]-iododeoxyuridine-labeled target cells mixed with effector cells were used as a measure of cytotoxicity. Among various target cells examined, only RBL-3 (a Rauscher virus induced, non-producer leukemia of BL/6) and $EL4G^{-}$ (carcinogen-induced lymphoma of BL/6) showed a high (20-30%, and 10-20%, respectively, above normal control level) cytotoxicity. Among various effector cells examined, only D4 and two other established SJL/J RCN, i.e. RCS5 and RCS19 (obtained originally from Sloan-Kettering Institute) were found to exhibit a high killer activity. Primary RCN and transplantable lymphocytic leukemias of T-cell origin gave negative results.

The killing effect was also confirmed by in vivo transplantation experiments. For this, EL4G$^-$ target cells (10^6) were mixed with RCS5 cells as effector cells at effector:target ratios of 200, 100, 50 and 25. Normal SJL/J spleen and lymph node cells were used in place of RCS5 as controls. After cocultivation for 20 hrs, the mixtures of target and effector cells were injected subcutaneously into C57BL/6 adult mice. The evidence that the resulting tumor is due to the growth of EL4G$^-$ cells but not due to that of RCS5 is shown by the absence of tumor in another control group of C57BL/6 mice which received RCS5 only. The EL4G$^-$ cells treated with RCS5 at all cell ratios tested showed significantly lower tumorigenicity as indicated by smaller size and lower incidence of tumors as compared with the control groups.

The cytotoxicity of RCN is sensitive to UV, X-ray (10,000 R) and freeze-thawing, is not due to macrophages, is selective for certain targets, and is partially or scarcely inactivated by anti-Thy 1.2 serum plus complement. Under the same conditions of experiment, the cytotoxic activity of immune spleen cells derived from SJL/J mice immunized with RBL-3 (allogeneic immunization) or C57BL/6 mice immunized with RBL-3 (syngeneic immunization) showed 80% reduction after treatment with anti-Thy 1.2 serum plus complement, whereas with D4, RCS5, and RCS19 as effector cells, there were 15, 47, and 54% reduction in cytotoxicity activity after similar treatment. Such a partial expression of Thy 1.2 antigen is in accordance with the findings of Herberman et al., that natural killer cells derived from normal mice of various strains are partially Thy 1.2-positive.

In order to examine the question of host cell participation in the cytotoxicity activity exhibited by RCN which grew in spleens and lymph nodes after transplantation, (C57BL/6xSJL/J) F_1 mice were used so that the host cells in the RCN tumor preparation could be removed by treatment with anti-$H2^b$ serum plus complement. However, presumably because of Hh (Hemopoietic-histocompatibility gene) restrictions, the RCN of SJL/J origin could not grow in (C57BL/6xSJL/J)F_1, or other semisyngeneic F_1 hybrids. It was found, however, that pretreatment of these F_1 mice with 5-10 mg silica particles to block macrophages will partially abrogate the F_1 resistance against RCN transplant growth. The cytotoxic activity of RCN from these treated mice could be reduced by 60% by in vitro pretreatment of RCN suspension with complement and anti-$H2^b$ serum in the case of C57BL/6xSJL/J) F_1, or anti-$H2^d$ serum in the case of (BALB/cxSJL/J) F_1 mice. Under the same conditions, the cytotoxic activity of RBL-3-immune F_1 mice was reduced by 70%. These results indicated that host cells are involved in the cytotoxic activity of RCN.

Thus, established RCN of SJL/J mice exhibit a markedly elevated natural killer cell activity which may be due to mobilization or induction of differentiation of host natural killer cells, or alternatively, due to the cooperation of host cells and RCN as a subpopulation of natural killer cells since it appears that the presence of RCN cells in the system is a necessary but not sufficient condition for manifestation of cytotoxicity.

Further work is necessary to clarify these points, and to define the surface antigens of RBL-3 recognized by the natural killer cell activity of RCN. In view of the similarity of the effector cells responsible for Hh restriction with those responsible for natural killer activity as reported by R. Kissling et al., the natural killer activity exhibited by RCN of SJL/J mice deserves further investigation.

IV. A virus-nonproducer leukemia cell line, RBL-3, originally transformed by Rauscher leukemia virus in C57BL/6 mice has been described. It appears that there exists a cellular block in the later stages of virus maturations, e.g. a defect in the cleavage of precursors of viral structural proteins or in the assembly process. As compared with a virus-producer leukemia cell line, RBL-5, similarly induced by Rauscher virus, there was a more abundant membrane expression of p30 and gp69/71 in RBL-3 cells, because of accumulation of viral products before assembly of complete virions. Recently, it was found, with the cooperation of Dr. G. S Aulakh, that RBL-3 has a greater amount of RNA which hybridizes with the complementary DNA (c-DNA) prepared from Rauscher virus, as compared with RBL-5. This fact is concordant with the above hypothesis that the cellular block is at the later stage of virus maturation, but the greater amount of RNA could be due to greater number of integrated c-DNA (provirus) or greater efficiency of transcription, or alternatively lesser degrees of degradation.

The FMR-specific transplantation antigen present in RBL-3 cells has been described. The crude membrane (CM) and crude soluble (CS) preparations also expressed the FMR-specific transplantation antigen. It was found, however, that semisyngeneic mice immunized with greater doses (400-800 μg) of CM rejected the challenge transplant of RBL-5 less effectively than those immunized with lower doses (25-50 μg). Such an immunosuppressive effect of greater-than-optimal doses of CM for induction of FMR-specific transplantation resistance has been repeatedly observed. This phenomenon is currently under investigation.

It was also found that the $EL4G^-$ cell line (a carcinogen-induced tumor) when treated with Mitomycin-C and injected into syngeneic or semisyngeneic mice, at weekly intervals for 3 times, could induce a transplantation resistance against itself, but not against RBL-5. A partial resistance against RBL-5, however, was found when the survivors from the above experiments were re-challenged with RBL-5. On the other hand, mice immunized with RBL-5-CM were resistant to $EL4G^-$ transplantation. The nature of the cross-reacting antigen is being investigated.

V. The three subgroups (A, B, and C) of feline retrovirus show differences in their host ranges when heterologous cell cultures are tested for their ability to replicate these viruses. Advantage was taken of the fact that cell cultures of guinea pigs are suseceptible to subgroup C but resistant to subgroups A and B of feline retroviruses. An inbred strain of guinea pigs (strain 2) carrying a syngeneic tumor induced by a feline sarcoma virus (FeSV) pseudotype of subgroup C feline retrovirus, FeSV (FeLV-C), would provide a system for the study of relationship between viral antigens and

FOCMA (feline oncovirus associated cell membrane antigen). For this purpose, cat cells were first injected with FeLV-C and Sneyder-Theilen-FeSV which contains FeLV-A and B as helper viruses. The mixed product would be FeSV pseudotypes of FeLV-A, FeLV-B, and FeLV-C in addition to these helper viruses. By passage through strain 2 guinea pig cells, the FeLV-A and FeLV-B helpers and pseudotypes were eliminated. The guinea pig cell transformed by FeSV (FeLV-C) continued to produce the virus, and induced tumors when injected into guinea pigs. These tumor-carrying guinea pigs produced antibodies against FOCMA as well as viral gs and envelope antigens. By absorption tests with appropriate antigens it was shown that FOCMA was distinct from these viral structural antigens.

Objective: 3

Approach: 2, 4, 5

Publications:

Chang, K. S. S.: SJL/J mouse reticulum cell neoplasms exhibiting "killer" activity. In Advances in Comparative Leukemia Research. Elsevier/North-Holland Biomedical Press, 1977 pp. 327-330. Bentvelzen *et al*., Ed.

Chang, K. S. S.: Immune reactivity of RBL-3, a nonproducer leukemia induced by Rauscher murine leukemia virus in C57BL/6 mice. J. Natl. Cancer Inst. (in press) 1978.

Log, T., Chang, K. S. S. and Sarma, P. S.: Production of antibodies against feline leukemia and sarcoma viruses and the feline oncovirus associated cell membrane antigen in strain 2 guinea pigs. Virology (in press) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05552-09 LCBGY

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mammalian Cellular Genetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION

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SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to conceive, conduct and communicate the results of original research in the area of genetic manipulation of mammalian cells using the techniques of cell fusion, cellular hybridization and cybridization to produce unique material for the molecular genetic analysis of cellular events. Of prime importance in the laboratory now is the genetic system in mammalian mitochondria. The mapping of recombinations between mitochondrial DNA molecules is being studied using restriction enzymes and cRNA-DNA hybridization. The several distinctive subspecies of mouse are proving to be adequately marked material for such studies. Transformation to chloramphenicol resistance of chloramphenicol sensitive cell strains by mitochondrial DNA from chloramphenicol resistant mutant cell strains is being explored as a means to introduce new genes into mammalian cells so that the impact of such genetic surgery can be evaluated. We are also following the heritable consequences on cellular phenotypes of cytoplast fusion. Of special interest have been 1) the malignant phenotype and 2) the differentiated functions of normal thyroid cells. We are attempting to use direct microsurgical intervention (microinjection) in order to understand these phenomena.

Introduction

The study of mammalian mitochondrial DNA (mt-DNA) as a genetic unit has given us the possibility to develop and exploit selective systems for at least these cytoplasmic genes. While one half of our effort is still concerned with the formal molecular genetics of mammalian mt-DNA, the second half of our effort attempts to capitalize upon this expertise and to begin to explore the possibilities of using combinations of cytoplasmic masses without nucleus called "cytoplasts" with whole cells whose physiology may be somehow permanently reprogrammed or even regulated as a result of such fusions. These cytoplasmic hybrids, termed "cybrids", are being explored in a number of different laboratories as the most exciting new experimental probe for what are coming to be known as "cytoplasmic regulatory substances". From the earliest days of cell fusion experimentation, it has been clear that when nuclei become embedded in the cytoplasm of another cell that the "signals" or regulatory imperatives of the cytoplasm were able to effect profound changes in the synthetic behavior of the foreign nuclei. A classic example of this behavior is the reactivation of the avian red cell nucleus in the cytoplasm of any actively dividing cell. More recently with the new found ability to select for cytoplasmic markers in "genetic crosses", it has become possible to show that many of these regulatory elements do indeed reside in the cytoplasm and furthermore, that they may have heritable consequences of their action. Thus, the fusion of a fibroblast or neuroblastoma cytoplasm to a murine erythroleukemia (MEL) cell frequently results in permanent extinction of the MEL cells' capacity to initiate inducible hemoglobin synthesis on the part of the fused cells as well as all their progeny. Such permanent, heritable consequences of cytoplast fusion are not limited to negative control: in a work soon to be published, Goplalakrishnan has shown that fusion of the cytoplasm from a cell that does synthesize phenylalanine hydroxylase (PAH) is able to cause a cell line that did not synthesize this enzyme but which did possess the genetic capacity to do so, to initiate PAH synthesis and to preserve that function among all its progeny.

These experiments and others like them, going on in cell biology labs around the country, suggest a renaissance of classic regulatory or "developmental" biology and promise a new level of understanding, perhaps even a molecular solution to these perennial mysteries.

In particular, our unit will continue its preeminent position in mammalian mt-DNA genetics and will explore selected examples of regulatory events in cybrid cells (most notably in the rat thyroid system to be described below). But it is not enough to join the experiments being done in a number of labs, we must strive to make major new departures in approaching these problems. I have chosen to try to explore further the cytoplasmic regulatory elements at work in our cellular genetics experiments by developing a direct microinjection assay for these substances. Thanks largely to the work of Dr. Elaine Diacumakos, it is now possible to make micropipettes with which one may deliver intracytoplasmic and/or intranuclear injections of experimental materials and to expect that 80% or more of the injected cells will survive the operation and continue to divide. Such a technique would permit the development of an assay for regulatory substances and hopefully become the basis for ultimate purification and isolation of such substances. We have set up such a laboratory and have undertaken training and initiated a collaboration with Dr. Elaine Diacumakos of the Rockefeller University, New York, to these ends.

What in the last few years may have seemed to some to be a rather esoteric undertaking in mammalian mt-DNA genetics has provided the basis and launching point for an entirely new departure to the study of mammalian regulatory biology. The future looks bright indeed.

I. MOLECULAR GENETICS OF MT-DNA

Transformation by CAP-r mt-DNA

In the past year we have extended our initial exciting results showing that chloramphenicol resistance (CAP-r) could be transmitted to chloramphenicol sensitive (CAP-s) cells by treatment with purified mt-DNA alone. We (Drs. Ching Ho and Coon) have tried to decide if the mechanism of this "transformation" event were by 1) repopulation of the whole CAP-s cells mt-DNA by the entire CAP-r molecule, or 2) by a recombination-like event (similar to the incorporation and stabilization of antibiotic resistance markers in Pneumococcus). We have used subspecies of the laboratory mouse, Mus musculus musculus, and M. m. molossinus, and a closely related species Mus caroli. All of these mt-DNA molecules are easily distinguished using restriction endonuclease mapping (see our last year's annual report). In short, our results have shown that in 4 out of 4 transformed cell strains examined, there was no evidence of persistent CAP-r mt-DNA still present in the treated cell lines. This result implies strongly that a recombination-like mechanism supersedes when CAP-r mt-DNA of one subspecies is used to "rescue" a CAP-s cell strain of another subspecies. Our first efforts at examining these transformants at higher resolution (endonucleases yielding 27-35 fragments from a single mt-DNA molecule) for evidence of changed restriction sites has shown that one of the four examined has at least one changed restriction site. We have also established during the past year that all of the "transformants" are themselves capable of transmitting the CAP-r phenotype by cytoplast fusion and hence they do represent changes in the cytoplasmic markers.

Intersubspecific cybrid and hybrid crosses

Parallel to these studies of the mt-DNA mediated transformation we have initiated cybrid and hybrid experiments between the same sets of subspecies examined in the previous experiments. One of these KYK (M. m. molossinus) a CAP-s cell line that has received the cytoplasts of OC10, a CAP-r, M. m. musculus cell strain has been found surprisingly to show 50% molossinus mt-DNA and 50% musculus mt-DNA after 40-60 cell generations of CAP selection. This result was surprising because we had believed that the two subspecies were fully compatible as far as the mitochondria were concerned. Now, however, at equilibrium under CAP selection it is apparent that KYK (molossinus) cannot live with musculus (OC10) mt-DNA alone; it must preserve its own kind for some mysterious reason. Other evidence suggests that in this particular cell strain we have encountered an interdependence of mt-DNA functions that may lead us to the first nuclear genes to be found that are involved in the reglation of mt-DNA synthesis.

Although there are several purely genetic experiments that are being explored with these heterosubspecific cybrid cell lines that would be too involved to chronicle here, the cell lines may allow us to make a very convincing demonstration of the fact that CAP-r and mt-DNA segregate together (are indeed conincident). Our experiments have shown us that when such cybrids are cultivated for a long time in the absence of CAP, the selective agent, then the musculus CAP-r mt-DNA molecules may be lost from many subclones in the population. Such segregants have also lost the CAP-r phenotype. In cases where the cells have retained the CAP-r phenotype they also have the musculus mt-DNA. This kind of syntenic segregation analysis is used extensively to establish chromosome assignments in the human karyotype using human-mouse segregating hybrids.

Nuclear DNA "transformation"

We believe that the apparent ability of sheared nuclear DNA preparations to cause CAP-s cells to become CAP-r is a non-specific mutation-like event resulting from the (possibly random) incorporation of nuclear DNA sequences into mt-DNA. Such an hypothesis is testable since it should be possible to find inserted regions of non-mt-DNA in the mt-DNA molecules. These are several methods that permit such analysis: 1) restriction endonuclease patterns may show this directly, 2) in a heteroduplex molecule made by annealing the H strand of suspected DNA to the L strand of standard DNA (or vice versa) an unpaired region (representing the inserted, non-homologous nuclear DNA) should be selectively subject to S1 nuclease; with the single stranded region digested a new set of restriction patterns will appear because of the new cut introduced by S1; such cuts should not appear in control (non-heteroduplex) molecules, 3) such unpaired regions of heteroduplex molecules may be visible directly by electron microscopy. We shall try methods #1 and 2 first during the coming year.

Novel methods for introducing whole mt-DNA into mammalian cells

As we have proposed in the past, it may be of more than academic interest to learn to manipulate mammalian plasmid DNAs for cloning and as sites of potential genetic prosthesis. As mentioned above, we have not found the mt-DNA of one subspecies incorporated directly into another cell. We propose to couple these investigations of mt-DNA genetics and our microsurgical experiments and try: 1) direct, intracytoplasmic injection of mt-DNA (CAP-r, musculus mt-DNA into/CAP-s molossinus cells) and 2) treatment of isolated mitochondria with mt-DNA followed by injection of the treated mitochondria themselves. This work is being done in collaboration with Dr. Elaine Diacumakos, of the Rockefeller University, New York City. The most simple and direct, straightforward demonstration of our ability to do intracytoplasmic injections may be simply to place CAP-r mitochondria into the cytoplasm of a CAP-s cell and then follow its selection in CAP. These experiments were attempted in January and will be reattempted in June.

It is hoped that either or both of the methods of injection of whole mt-DNA molecules into cells will succeed. In any event, they should provide further direct evidence (at least in the case of the injected whole mitochondria) that the CAP-r gene is indeed a genuine mitochondrial marker.

Other plans for the coming year

We shall take up W. French Anderson's (NHLB) lab's offer to make available P3 facilities and their expertise in cloning DNAs both in the *E. coli* plasmid vectors and in lambda. We plan to make clones of several of our CAP-r DNA's and some of the putative recombinant and "transformed" molecules. The larger quantities available through the cloning techniques will be crucial for our projected more detailed analysis of mt-DNA genetics.

Using microinjection techniques, we shall attempt to determine if human mt-DNA will replicate as an episome in mouse cell lines. The hope is to inject human mt-DNA into the mouse cell lines' nucleus. Human mt-DNA can be isolated independently from mouse mitochondrial or nuclear DNA with CsCl gradients and cRNA probes are unequivocal after such a preliminary separation, thus making the experiment eminently feasible. If mt-DNA does replicate as an episome in the mammalian nucleus, then this study would shed new light on foreign DNA replication in the nucleus as well as provide a new approach to the problem of cloning DNAs in mammalian cells.

II. REGULATORY BIOLOGY

The newer aspects of our approach to this topic have been the subject of a separate report, "A Proposal to Expand the Efforts to Study the Heritable Consequences of the Fusion of Normal Cell Cytoplasm with Specialized and/or Malignant Cells" that was submitted on 7 December 1977 and will only be outlined here.

Cybridizations: current experiments

We have been interested in two questions that may well be related: 1) the regulation of the malignant state and 2) the regulation of differentiated functions in both normal and malignant cells. Nearly three years ago we proposed examining these questions by cytoplast fusion. Specifically, we ask: does the cytoplasm of a normal cell contain whatever cytoplasmic regulatory substances may be needed to regulate the growth control mechanism(s) in a malignant cell? Our hypothesis states that an important feature of the malignant cell phenotype is a mis-regulation of the normal growth control mechanism. We seek to reregulate this misregulated function by supplying missing regulatory factors via cytoplast fusion. In order to have more than transitory effect, such factors would have to be of the autogenous or self perpetuating (not necessarilly self replicating) kind. At this writing, May, 1978, our experiments are not yet complete; however, we know that Howell and Sager claim in a forthcoming paper that this result is possible. That is, that a normal cytoplast fused to a malignant cell yields a non-malignant cybrid cell strain. [Malignancy is here tested by growth or failure of growth in nude mice.] We have chosen two rat cell systems in which we have considerable experience, Buffalo rat liver cells (BRL) and Fischer Rat Thyroid cells (FRT). In each case we have very excellent control over the cell biology of the system because we have tumor cells and absolutely normal cells growing in culture under serum free conditions. Because our first experiments to be completed will be with the thyroid system, we shall discuss that system in more detail.

I-5G and FRA are experimentally induced tumors of the thyroid origin that are serially transplantable in Fischer rats. FR-T and FR-TL are normal cells that we have cultured in addition to the tumor strains. One of these, FR-T, has been grown under conditions previously considered standard for colonal culture of rat epithelial cells. Although FR-T is normal, i.e., it does not produce tumors in Fisher rats or nude mice, it does not produce thyroglobulin (TGB) or concentrate iodide from the medium as freshly isolated thyroid cells do. The other cell line, FR-TL has been grown in our new serum free, hormone supplemented 6H medium. These cells, too, are normal (they do not produce tumors) but they are fully differentiated and normally responsive to hormones (particularly TSH) in culture. The FR-TL cultures obtained in collaboration with Dr. F. Saverio Ambesi-Impiombato during his visit in our laboratory, represent a significant advance in the culture of normal differentiated cells.

Cybrids of the normal cells have been fused to the malignant strains. We are awaiting cybrids for testing in nude mice for expression of the malignant phenotype. And since the malignant lines (FRA and I-5G) have also lost their differentiated functions of TGB synthesis and I_2 concentration, the FR-TL (cytoplast) x FRA experiment will also test the ability of the normal cells' cytoplasm to reregulate the missing differentiated functions as well.

Microinjections: the prospects

In all such experiments in somatic cell genetics such as hybridizations and cybridizations the problem comes up: what next? These techniques seem able to generate limitless supplies of intriguing phenomena but precious little in the way of hard understanding, i.e., molecular mechanism. We must, therefore, try to penetrate the phenomena and try to fashion the basis for an analysis. I have chosen to do this by adapting the techniques of microinjection to this kind of search for cytoplasmic regulators. Our first experiments will ask simply: can we reproduce the results of a cytoplast fusion by injection of the cytoplasm (or a cytosol extract) of one cell directly into the cytoplasm of another whole cell? If the answer to this question is yes, or even only partially yes, then we may have established the basis for an assay of regulatory substances that should make possible their purification and isolation. We envision, sometime in the coming year taking the cytosol extract of an FR-TL cell and microinjecting that into several FRA cells. These FRA cells will be isolated and grown up and tested for 1) malignancy (ability to grow in nude mice); 2) reexpression of the missing differentiated functions.

These experiments represent an important new departure for the Cellular Genetics Unit as well as the Laboratory of Cell Biology itself. We are for the first time doing experiments that seek directly to alter the malignant phenotype by reestablishing what may be normal growth regulation patterns.

Publications:

Coon, H. G. and Ho, C.: Transformation of cultured cells to chloramphenicol resistance by purified mammalian mitochondrial DNA. In: Genetic Interaction and Gene Transfer. Brookhaven Symposia in Biology: No. 29 pp. 166-177, 1977.

Kidson, C., Moreau, M-C, Asher, D. M., Brown, P. W., Coon, H. G., Gajdusek, D. C. and Gibbs, C. J. Jr.: Cell fusion induced by scrapie and Creutzfeldt-Jakob virus-infected brain preparations. Proc. Natl. Acad. Sci. (in press) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05553-09 LCBGY

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Structure of Mouse Myeloma Proteins with Antibody Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Immunochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing studies of the last year have dealt with the determination of the primary and three dimensional structure of antigen binding myeloma proteins. Two crystallized immunoglobulins with specificity for phosphorylcholine and galactan are currently under investigation. In addition, groups of proteins with these and other binding specificities are being examined to identify patterns of variation which will in turn reflect genetic mechanisms involved in the generation of antibody diversity.

Studies have been initiated to attempt to ascertain if light chain variable regions are a component of thymocyte receptors. Antisera specific for light chain variable regions are currently being assayed for activity against T cells.

Studies are in progress to chemically characterize major histocompatibility antigens in inbred miniature swine. We are attempting to isolate these antigens for sequence analysis and eventually employ them in graft suppression experiments.

PHS-6040
(Rev. 10-76)

Project Description:

A. During the past year we have continued studies of the primary and three dimensional structure of the antibody molecule. This project has involved the analysis of groups of antigen binding myeloma proteins identified by Dr. Michael Potter which have been shown to have antigen binding activity for a series of chemically defined haptens including phosphorylcholine, galactan, and levan. The comparative study of the three dimensional structure of proteins with differing antigen binding specificities will permit an evaluation of the types of topographical changes which occur in antibody molecules to account for the structural basis of the vast degree of antibody diversity. The determination of the primary amino acid sequence of these and their naturally occurring counterparts, in addition to being essential for the three dimensional analysis, will further provide an insight into the genetic mechanisms involved in the generation of antibody diversity.

1. Previous studies on phosphorylcholine binding myeloma proteins have resulted in the crystallization and determination of the three dimensional structure of one of these proteins. These data combined with the primary amino acid sequence of the heavy chain permitted the construction of a molecular model of this antigen binding site. We have recently completed the primary sequence of the light chain from this molecule. Crystallographic data are now in the final stages of refinement, and together with the new primary sequence data should in the near future produce a final molecular model defining the basic interactions occurring in this protein and in its interaction with antigen. We have also now completed the light chain sequence of a second phosphorylcholine binding protein and are currently analyzing the patterns of diversity exhibited by these light chains in comparison to that of other kappa chains as well as our previously reported data on the heavy chains from three of these proteins.

2. The germ line nature of the immune response to phosphorylcholine has been established by a variety of studies. The recent introduction of the hybridoma technique in which a plasma cell tumor line is fused with normal spleen cells and the resultant cell continues to make the product of the spleen cell but grows 'immortally' as a tumor, now permits us to look structurally at the products of a 'normal' B cell and compare these proteins to those found in the myeloma tumors. Hybridomas have currently been made to phosphorylcholine from both BALB/c and other strains. We shall analyze a series of these products to ascertain the pattern of variation, the relation to similar proteins from myeloma tumors, and the genetic polymorphism as reflected in the secreted immunoglobulins.

3. One of the longstanding questions in immunology is concerned with the basis of the generation of antibody diversity. It is still unclear as to whether the majority of diversity is encoded in separate germ line genes or if somatic mutation occurring on a relatively few germ line genes can account for the vast array of antibodies. While this laboratory and others have accumulated evidence for an increasing number of germ line genes, these two

hypotheses are not mutually exclusive, and it seems quite likely that both participate in the generation of diversity. We are attempting to analyze the effect of a possible somatic mutation mechanism in the following system. Dr. M. Scharff has placed a line of a phosphorylcholine binding myeloma protein in long term tissue culture. Spontaneous mutants have arisen from this line which bind the antigen to a much lower extent but in other characteristics are identical to the parent line. A series of revertants and further mutants have subsequently been isolated from the original mutant. The hypothesis is that the variants are likely to result from single amino acid substitutions as would be expected to arise from a somatic mutation process. Structural analysis of these clones should establish the basis of the binding mutations. This phenomenon can also be analyzed *in vivo* by the isolation of hybridomas with characteristics of the parent line (idiotype) but decreased antigen binding capacity.

4. Another problem related to diversity currently being explored is that of the heterogeneity of the antibody response to protein antigens. To date very little is known about the structural basis of the immune response to protein antigens while these determinants along with carbohydrates are probably the most prevalent in nature. We are currently attempting to analyze the structural heterogeneity of the immune response to Staph. nuclease by isoelectric focusing. We have developed a transfer technique by which heavy and light chains are first separated and then electrofocused to analyze light chain heterogeneity. We are also attempting to develop a radioactive antigen overlay to assess heterogeneity of intact molecules and to evaluate polymorphism among inbred strains.

5. As mentioned above, proteins and carbohydrates are probably the most prevalent antigens in nature. We have also studied two groups of myeloma proteins with binding activity for the carbohydrates (2 1) levan and (1 6) galactan. Details of the anti-levan studies were presented in the preceding Annual Report. Analysis of light chains from four galactan binding myeloma proteins has established that this group of molecules must be coded for by at least one germ line gene and the variation among these light chains is minimal as in the case of the kappa chains from levan binding myeloma proteins. Evidence also suggests the probability of multiple genes coding for at least part of the constant region.

The heavy chains from the galactan binding myeloma proteins have presented difficult problems in terms of peptide isolation. Experimental procedures have been developed in the last year which have now permitted isolation of the appropriate fragments. Nearly complete sequences are available for three heavy chains from this group and a fourth is currently in progress. The completion of the galactan sequences will be the final segment of our study on groups of antigen binding myeloma proteins. At this point we are now analyzing our accumulated data from phosphorylcholine, levan, and galactan binding proteins in terms of the compatability of the patterns of variation observed with the mechanisms proposed for generation of antibody diversity. The galactan binding proteins are of further importance in that two of these have been crystallized and X-ray crystallographic studies are currently in progress on these molecules. The X-ray data combined with the primary sequence analysis will permit construction of a molecular model which can then be compared with the previously determined structure of the phosphorylcholine binding protein M603.

B. Recent studies of receptors on thymocytes have indicated that these molecules can be antigen specific and possess the same antigenic determinants (idiotypes) as classic antibodies. These receptors have been shown to have structural similarity to the variable regions of immunoglobulin heavy chains but no constant region determinants or light chain determinants have been detected. Considerable controversy therefore exists as to the molecular nature of this receptor.

1. In view of our studies on the structure of the antibody molecule, we feel it unlikely that this receptor is composed solely of heavy chains. We are therefore analyzing T cells for the presence of immunoglobulin light chains in the following manner. Since both light and heavy chain constant regions are undetectable on T cells, this part of the molecule must be either absent or inaccessible to the specific reagents. We have therefore prepared rabbit antisera to the light chains of myeloma proteins with phosphorylcholine binding activity. We have previously shown that these light chains are also present in the induced immune response to phosphorylcholine. The antisera are then made specific for the light chain variable regions by appropriate absorption. We are currently assaying these reagents for activity on T cells by the fluorescence activated cell sorter and by *in vitro* inhibition of phosphorylcholine specific T-cell proliferation.

C. In recent years a considerable effort has been devoted to studies of the major histocompatibility complex in terms of graft rejection and a variety of other immune functions. In collaboration with Dr. D. Sachs we have begun studies on the chemical characterization of the major histocompatibility antigen from inbred miniature swine. These animals are particularly attractive as a model system in that their organs are of the same approximate size as humans. A large transplantation program is currently in progress using this system. The long term aim of this project will be to chemically characterize the major histocompatibility antigen involved in graft rejection and then use either the natural or synthetic products to attempt to suppress graft rejection.

1. Experiments have been initiated to purify swine histocompatibility antigen for chemical studies. Histocompatibility antigens have been shown to consist of two polypeptide chains designated as heavy (40,000 M.W.) and β_2-microglobulin. Human transplantation antigens have been purified specifically by the use of antisera to β_2-microglobulin. β_2-microglobulin from the human has been shown to exhibit cross-reactions with swine β_2. We are therefore in the process of attempting to use appropriate anti-human antisera to isolate swine β_2. We will then make antisera to the swine β_2 which will be used for purification of histocompatibility antigen.

Publications:

Claflin, J.L. and Rudikoff, S.: Uniformity in a clonal repertoire: a case for a germ line basis of antibody diversity. Cold Spring Harbor Symp. Quant. Biol. 41: 725-734, 1976.

Morse, S., Goode, J. and Rudikoff, S.: Murine plasma cells secreting more than one class of Ig heavy chain. IV. Sequence differences between kappa chains of 5368 IgG2b and IgA. J. Immunol., 119: 361-363, 1977.

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Rudikoff, S. and Potter, M.: Kappa chain variable region from M167, a phosphorylcholine binding myeloma protein. Biochemistry (in press), 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05596-09 LCBGY

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Pathogenesis of plasma cell neoplasia in mice: immunochemical characterization of antigen-binding myeloma proteins; genetic basis of immunoglobulin diversity.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION

Immunochemistry

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SUMMARY OF WORK (200 words or less - underline keywords)

1. Mechanisms in mineral oil plasmacytomagenesis in the BALB/c mouse are being actively pursued. The role of the oil granuloma, microenvironmental dependency is being studied in vivo in nu/nu mice, and an in vitro model is being developed. Attempts are being made to grow human myelomas in pristane conditioned nu/nu mice. Work on the determining genetic basis of susceptibility continues with the development of a number of BALB/c - DBA/2 congenic strains, in which all available DBA/2 markers are put on the BALB/c background. The role of the LPS-C3H/HeJ gene, the nu/nu defect, are also being tested. Attempts to isolate plasmacytomagenesis type C viruses is underway. An in vitro method of plasmacytoma induction is in progress using Abelson virus.

2. Antigen binding activities of myeloma proteins continues with a search to find myelomas with anti-idiotypic activity. Myeloma proteins are being screened with 250 other myeloma proteins to find interacting proteins (anti-idiotypes, anti C-region markers).

3. The study of individual idiotypes of 8 galactan binding myeloma proteins indicates XRPC24 may be the germ line gene, and the other 7, possible somatic mutants.

4. V-kappa isotypes are being defined by amino acid sequences. 33 different isotypes among 78 proteins have been found in BALB/c. The VK-21 family of closely related chains is controlled by at least 5 genes. The VK-genome size and structure may be estimated in the BALB/c from sequence data.

5. Search for allelic V-region markers continues.

Plasmacytomagenesis

We continue to characterize the conditioning (promoting) effects of the mineral-oil granuloma. The peritoneal oil granuloma is an essential growth condition for transplantation of primary plasmacytomas in BALB/c mice in the peritoneum. This dependence is rapidly lost during continuous transplantation. Efforts to develop an in vitro assay for the dependency to independency conversion is in progress. When available attempts will be made to determine the underlying biochemical changes, assays for plasminogen activator, prostaglandin production, will be studied.

Continued study of conditioning in vivo will include studies to determine if T-cells play a role. First, it will be determined if pristane or mineral oil injected nude mice (which lack T-lymphocytes) can, support the growth of primary plasmacytomas. Three primary tumors transplanted so far into BALB/c nu/nu mice have shown dependency on the oil-granuloma. One tumor which is dependent in BALB/c was independent in nu/nu.

We shall also attempt to transplant human myeloma cells, possibly lymphosarcoma cells into conditioned nu/nu mice, to determine if human plasmacytoma cells also have a similar requirement for an adherent cell. The apparent origin of multiple myeloma in the bone marrow cavities strongly suggests a local micro-environment supplies an essential factor for growth. Possibly other tissue components will have to be supplied to nu/nu hosts, and this could include bone marrow stroma. Arrangements have been made with Dr. John Minna, NCI, to obtain cells for these studies. We have currently established a nu/nu mouse colony, suitable for long term maintenance.

Abelson Virus (MLV-A)

We continue to study the role of the defective Abelson retrovirus as an initiator in plasmacytomagenesis. In collaborative studies with Dr. Charles Scher of the Sidney Farber Cancer Center, Boston, Ma, we are studying the plasmacytomagenic properties of MLV-A in vitro. Cells from CB-20 mice are infected with MLV-A and stimulated with LPS. After varying intervals the cells are transplanted into BALB/C mice. The myeloma proteins produced by plasmacytomas that develop in the BALB/c are allotyped. We have been able to induce one tumor in 8 days in culture. This method is very promising and could lead to the identification of the MLV-A target cell type. We shall explore Peyer's patch, peritoneal exudate, mesenteric node and spleen cells as a source.

Attempts to Rescue Abelson-like (transforming) Viruses from BALB/c Mice

In collaborative studies begun with R. Lerner (Scripps Institute) and continuing with J. Hartley (NIAID) type C viruses have been isolated from plasmacytomas in early transfer generations including one duotropic MCF type virus, and several N- and B ecotropic. Early transfer generation plasmacytomas have been successfully superinfected with Moloney virus (the rescuing agent for MLV-A) (in collaboration with C. Scher) extracts from these tumors have been injected into pristane conditioned BALB/c mice. Three tumors are currently being studied. Early developing leukemias will be studied for the presence of transforming viruses.

Plasmacytomagenic Properties of MCF Virus from CBPC208

In collaboration with J. Hartley NIAID we are testing the duotropic MCF (mink cell focus forming) retrovirus for plasmacytomagenesic activity both by the *in vitro* infection method, and by direct *in vivo* inoculation.

Isolation of Type C Retroviruses from BALB/c Mice during Pristane Plasmacytomagenesis

In collaboration with J. Hartley NIAID adherent peritoneal exudate cells, from pristane primed BALB/c mice are being systematically studied for retrovirus production. Viruses can be isolated from adherent peritoneal cells within the first few weeks after pristane. Adherent cells are being studied from mice in the pre-plasmacytomagenic period and, the types of viruses obtained from adherent cells will be compared with those obtained from the primary plasmacytomas that develop in these mice.

Genetic Basis of Susceptibility

Only two inbred strains BALB/c and NZB are thus far known to be susceptible to induction. The identity of genes involved in this process are not yet identified.

In collaborative experiments with Dr. Carol Cowing NCI we tested the susceptibility of a backcross progeny in a cross between BALB/c and the resistant DBA/2, which had been selected for allotype difference in Tol-1^{a} gene(s) that govern resistance to tolerization. This specific trait was chosen for study because both BALB/c and NZB are strains that are relatively difficult to tolerize. BALB/c are Tol-1^{bb}, and DBA/2 are Tol-1^{aa}. The incidence of plasmacytomas in the BC$_{2}$ mice was too low to determine the influence of the Tol-gene. It thus became apparent DBA/2 carries several resistance genes, and we began then to examine this more systematically. A number of BALB/c - DBA/2 congenic strains are being developed (most are currently of BC5) using the following DBA/2 markers: Chromosome 1, Id-1, D1P-1; Chr-2,a; Chr4, Fv-1;

Chr5, Pgm-1, B-Gus; Chr7,C; Chr9,d; Chr11 esterase-3, and unlocated, IgC_H (allotype), Tol-1; Hc-1; and others. BC_6 heterozygotes will be tested for plasmacytoma susceptibility to locate resistance (R) genes.

In addition we are constructing a BALB/c.C3H/HeJ congenic and selecting for the lps marker in collaboration with Dr. D. Rosenstrich NIDR. We will also test BALB/c nu/nu mice for susceptibility.

We continue to test other inbred strains ST/1 Ma/my, BALB/JAX, BC-8, C57L, PL, and AKR are currently under study.

Antigen Binding Myeloma Proteins Structural Studies

This laboratory has been intensively studying 5 groups of antibodies defined usually by their hapten binding specificities: phosphocholine (PC), B1,6 galactan (β6GAL) flagellin (Fla), B2,1 fructosan (Gβ1F) and B2,6 fructosan (Gβ6F). In collaboration with Dr. S. Rudikoff, Dr. M. Vrana, and Dr. N. Rao we have been determining the primary structures of the V_L and V_H regions: in collaboration with Dr. C. P. J. Glaudemans (NIAMDD) we have been studying the immunochemistry and specificity of hapten binding, and with Dr. E. Padlan and D. Davies (NIAMDD) we have been studying these proteins at the three dimensional levels. Currently it is possible to construct hypothetical three dimensional models of Fv fragments from primary structural data. In cases where crystals cannot be obtained, it is possible to confirm inter-atomic distances in the combining site of proposed structures using the technique of nuclear magnetic resonance. Dr. Raymond Dwek of Oxford has pioneered in this method and has confirmed the proposed structure of the MOPC315 binding site. At a recent visit by Dr. Dwek, we established a collaboration with the Oxford group to determine the contacting amino acids in J539 a galactan binding myeloma protein, EPC109 a B,2,1 fructan binding myeloma protein and to begin work on MOPC467 a flagellin binding myeloma protein (the only example of an anti-protein myeloma in our collection). The models of the first two proteins have been built by E. Padlan from the sequences obtained by Drs. Rudikoff, Vrana and Rao. These prototype molecules each have a very distinctive binding site with both marked secondary structural (contour) variations as well as a great difference in the array of amino acid side chains that project into the binding surface. The analysis of these proteins should provide the basis for a more complete understanding of antibody structure and specificity.

We have supplied myeloma proteins to Dr. D. Davies for crystallization studies. Both J539 and E109 have been crystallized and Dr. Davies' group is determining electron density maps and working models of J539.

Immunoglobulin-V-region Genetics

One of the continuing efforts of this laboratory has been to systematize the V-kappa isotypes of the BALB/c mouse. Fortuitously the amino acid sequence of the chains up to the first invariant Trp35 provides a means for typing and identifying germ line V_k-structural genes. The mouse has a great diversity of structures (33 V-kappa isotypes so far identified) which are expressed in the myeloma pool and many of these have been related to specific antibodies.

In collaboration with Dr. S. Rudikoff and Ms. J. G. Pumphrey, we continue to study V_k-isotypes by partial sequencing, 25 new kappa chains through Trp35. These data hopefully will define the extent of V-kappa genome in the BALB/c mouse. The mouse is an ideal species for studying kappa chain diversity because, for evolutionary reasons not understood, the framework region, FM-1, has many differences from one V-region to another. This is in contrast to human V_k in that only 4 to 5 FM-1 subgroups have been defined. We may therefore be able to estimate the V-region genome size in the mouse by primarily structural data.

In collaboration with Dr. D. J. McKean of the Mayo Clinic we continue to determine sequences on the VK-21 group of proteins. This group now contains 5 isotypes VK-21A to E. VK-21 isotypes may differ from each other by as few as 5 positions. Since an isotype is a minimal definition of a germ line V-gene, the BALB/c mouse genome contains a large number of V-identifiable V-K genes. The genetics of Vk genes offers exciting opportunities in the area of mammalian gene regulation. There is a great need to develop Vk markers in the mouse based upon homologous Vk differences. We hope to identify such markers using wild mouse genes from species of musculus e.g. M. m. molossinus and M. m. castuneus. Hybridomas of molossinus and castuneus with MOPC21 (P3) are to be made by S. Clarke, in collaboration with F. Melchers at the Basel Institute and these will be compared with homologues in BALB/c as a potential source of V_k markers.

We continue to study the β1,6 galactan binding myeloma proteins (GALBMP) and their relationship to β1,6 galactan antibodies. These proteins all have VK1B-Vk4 isotype composition. We (E.B. Mushinski) have induced antibodies to gum ghatti in mice and shown these antibodies share cross-specific idiotypes (IdX) with GALBMP. Each of the 8 GALBMP however, has a characteristic private or individual idiotype (IdI). BALB/c antibody by RIA analysis contains a large amount of XRPC24 IdI indicating the XRPC24 may be very closely related to the germ line gene. The regulation of the VH1B-Vk4 expression will be further explored this year.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08726-01 LCBGY

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biological and Biochemical Characterization of Transplantation Antigens.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. J. Rogers	Sr. Staff Fellow	LCBGY	NCI
OTHER:	O. Alaba	Staff Fellow	LCBGY	NCI
	E. Appella	Medical Officer (Res.)	LCBGY	NCI
	L. W. Law	Chief	LCBGY	NCI
	S. Oroszlan	Staff Scientist	Frederick Cancer Research Center	
	G. Parmiani	Staff Scientist	National Cancer Institute, Milano, Italy	

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.50	.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to examine the biological and immunological properties of histocompatibility antigens (H-2) and tumor specific transplantation antigens (TSTA) and, ultimately, to purify the molecules expressing these antigens. The approach is to purify both antigens from a single cell source (RBL-5) and determine their antigenic properties at each stage of purification. The antigenic properties monitored include: 1) ability to induce transplantation rejection, 2) ability to evoke a humoral response, 3) ability to interact with syngeneic or xenogeneic antisera. At the same time H-2 and TSTA with different biological properties from other sources are purified and compared with the antigens from RBL-5. The rationale for this approach is that through purification of the antigens from a single source one can learn the relationship, if any, of TSTA to H-2 and to what extent the properties of these antigens depend on the structure of the intact cell membrane. Furthermore, any unusual structural properties of H-2 and TSTA from other sources, when compared with the structure of their counterparts from RBL-5, may shed light on the molecular basis for the biological activity of these antigens.

PHS-6040
(Rev. 10-76)

Rogers, M. J., Prat, M., Appella, E. and Law, L. W.: A study of immune mechanisms in the rejection of syngeneic murine leukemia. Role of solubilized antigens from the plasma membrane. Proc. 6th Int. Quadrennial Cancer Conference 21: 246-252, 1978.

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Appella, E., DuBois, G. C., Natori, T., Rogers, M. J. and Law, L. W.: Histocompatibility antigens and tumor-specific transplantation antigens of a methylcholanthrene-induced sarcoma. In Proceedings International Symposium on Biological Markers of Neoplasia (in press) 1978.

Results:

I. Purification of TSTA and H-2^b from RBL-5 (Rauscher virus induced leukemia) Tumor Cells.

H-2^b and TSTA were isolated from highly purified plasma membranes of RBL-5 cells using detergent solubilization, lectin affinity chromatography and affinity chromatography on specific immunoabsorbants. The TSTA was clearly separated from H-2^b and from the major Rauscher viral structural also monitored proteins in this study. H-2^b was purified more than 1000 fold (~50% pure) and obtained in quantities sufficient for structural characterization and reconstitution experiments using artificial membranes. These artificial H-2^b membranes will be used to compare the biological properties of H-2^b on the membrane with soluble H-2^b. The purification of TSTA is being continued by preparing syngeneic and xenogeneic antisera against the most highly purified fractions which can be used for preparing immunoabsorbants.

II. Purification and Biological Characterization of TSTA from an SV40 and SV40 Induced Tumor, TU5.

The TSTA of this SV40 induced tumor is found primarily in the nucleus and, when cells are disrupted, remains in the high speed supernatant. The water solubility of the TSTA contrasts with the TSTA of RBL-5, which is membrane bound and can only be solublized with detergents. The biological properties of SV40 TSTA were examined to see if they differed significantly from those of RBL-5. The TSTA was partially purified by gel filtration before use. It was found that the soluble TSTA was specific and very active. Immunization with from 5 to 1000 μg gave complete protection. It has been suggested in the literature that soluble tumor antigens might play a role in the modulation of the anti-tumor immune response. Attempts to reduce or abrogate the immune response with high dose of this TSTA were unsuccessful. Furthermore, the behavior of the soluble TSTA did not differ significantly from the TSTA found on membranes of the same TU5 cells. Thus, the suggestion that soluble antigen can modulate the immune response does not seem to be born out in this system.

III. Immunological Properties of the RBL-5 TSTA.

In constrast to the results with TU5 cells, RBL-5 membranes and solubilized TSTA were found to be immunogenic only at low doses (5-100 μg). Excessive doses of the antigen (200-1000 μg) did not produce immunity. This was true for both direct tumor challenge, and Winn assay. These experiments are being continued by: 1) extending these results to an *in vitro* Cr^{51} release assay system, 2) performing mixed experiments in the Winn assay and adoptive transfer experiments to determine if a lymphocyte population is actively suppressing immunity, 3) determining if the antigen responsible for the suppression copurifies with the TSTA.

IV. Purification and Biochemical Characterization of an Alien H-2 Molecule from a Methylcholanthrene Induced Tumor.

The chemically induced tumor C-1 has been shown by Parmiani to express, besides the expected H-2K^d and H-2D^d specificities, an alien H-2 antigen, H-2K^k, normally expressed only on cells from animals of the k haplotype. In collaboration with Dr. Parmiani, we have undertaken to purify this antigen from the membrane of C-1 cells. So far a 20 fold purification has been obtained by conventional biochemical means, and the purification has been completed by radiolabelling and immunoprecipitation. The alien specificities have been shown to reside on a molecule distinct from the normal H-2 molecules of these cells. The alien molecule is similar to normal H-2 molecules in many respects. It has the same molecular weight on SDS gels (48,000); it is associated with β_2-microglobulin; and it binds to Lens culinaris lectin. Nevertheless, it differs from normal H-2 molecules in its susceptibility to papain digestion, anomalous elution profile from gel filtration columns and instability during storage. Moreover, it is present in 10-20 fold lower density on C-1 membranes than normal H-2. These biochemical differences may help to explain some of the anomalous biological properties of this antigen documented by Parmiani. The purification of this molecule is being continued so that sufficient quantities can be obtained for detailed chemical analysis.

Publications:

Liu, W. T., Rogers, M. J., Law, L. W. and Chang, K. S. S.: The properties of RBL-5 cultivated *in vitro*. J. Natl. Cancer Inst. 58: 1661-1664, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08727-01 LCBGY

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)
Organization and Control of Genetic Material in Immunoglobulin-secreting Plasmacytomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Mushinski	Medical Director	LCBGY	NCI
OTHER:	M. Potter	Head, Immunochemistry Sect.	LCBGY	NCI
	D. Hatfield	Research Biologist	CB	NCI
	D. Grunberger	Associate Professor	Columbia University	
	M. Marini	Research Associate	Univ. of Bologna	

COOPERATING UNITS (if any)

Columbia University and University of Bologna

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study the control mechanisms important in regulating protein synthesis in normal and malignant cells. Control mechanisms active in selecting immunoglobulin genes for transcription are being studied by comparison of nuclear non-histone proteins present in certain mouse plasmacytomas. cDNA probes constructed by reverse transcription of mRNAs will be used to locate active chromatin fractions in order to study the protein molecules bound to DNA.

The functioning of transfer RNA (tRNA) in plasmacytomas and other tumors has been studied by analytical column chromatography. Of special interest is the coding, structure and function of the tumor specific phenylalanyl-tRNA found in about one half of the mouse and rat tumors studied.

PHS-6040
(Rev. 10-76)

Methods Employed:

The study of nuclear proteins actively engaged in turning on particular Ig L & H chain genes or turning off all the other Ig genes will utilize DNA probes. Initial efforts are concentrated on isolating mRNAs from mouse plasmacytomas producing kappa chains of well characterized isotypes. Nuclear proteins of liver and plasmacytomas are being fractionated by electrophoresis for binding studies with DNAs.

Transfer RNAs from rat and mouse tumors including mouse plasmacytomas have been compared for all 20 amino acids using RPC-5 column chromatography. Computer methods have been developed to analyze these chromatographic data. Tumor specific Phe-tRNA found in a wide spectrum of rat and mouse tumors has been intensively studied by trinucleotide-directed ribosome binding and in vitro incorporation into protein in cell-free protein synthesizing systems.

Major Findings:

The studies of nuclear proteins have started with the investigation of a nuclear protein in mice that is apparently identical with the major mouse urinary protein, MUP. MUP has been quantitated in normal and tumor bearing mice using a radioimmunoassay. The presence of a growing plasmacytoma in the animal causes a decrease in MUP levels in liver cytoplasm and nucleus as well as in serum and urine. Another effect on liver metabolism from distant plasmacytoma growth is the appearance of a second isoaccepting species of asparaginyl-tRNA.

Visual analysis of the more than 250 chromatographic profiles of plasmacytoma aminoacyl-tRNAs has permitted grouping of these tRNAs into 2 groups, constant and variable, depending on whether liver and 11 tumors' tRNAs were similar or significantly variable. Comparison of aminoacyl-tRNA profiles with the structure of the secreted myeloma protein did not reveal a correlation. The tumors' aminoacyl-tRNA profiles frequently differ from those of normal liver suggesting that enzymes in the malignant tissues caused characteristic post-transcriptional modifications in tumor tRNAs. Analysis of the profiles by the computerized process of cluster analysis demonstrated that similar clonal origin of tumors resulted in similar tRNA profiles. The profiles remained the same even when drastic changes in myeloma protein synthesis occurred, such as cessation of heavy chain synthesis or complete lack of Ig synthesis. This important observation suggests that tumors such as these do not have the ability to vary their tRNAs when the protein synthesis of the cells changes. Such "functional adaptation" has been demonstrated for silk worms and sheep reticulocytes and had been thought to be a feature of all eukaryotic cells.

Tumor specific Phe-tRNAs are found to code nearly the same as the normal Phe-tRNA isoacceptor, mainly binding to UUC and UUU. A surprising finding was that the tumor Phe-tRNA appears to be incorporated faster into protein than the normal Phe-tRNA isoacceptor.

Significance:

The distant effects of malignancies are frequently seen in cancer cachexia in the clinic and in unexpected wasting death in experimental cancer. MUP has been described as a nuclear protein, (Ruoslahti et al., J. Exp. Med., 146, 1054, 1977) important in maintaining normal liver status since it is nearly totally absent in hepatomas. Our finding that plasmacytomas, even in the absence of metastases, cause hepatic MUP levels to decrease and a new isoacceptor of asparaginyl-tRNA to appear may provide a model to investigate the distant effects of cancer.

The survey of rat and mouse tumors indicates that tumor specific Phe-tRNA is present in more than half the tumors studied. The most significant aspect of this finding is that rat and mouse tumor-specific Phe-tRNAs differ in quantity and chromatographic elution position. This suggests that there may be different biochemical mechanisms at work in the two animals; e.g. rat tumor Phe-tRNA may be due to incompleteness of post-transcriptional modification and mouse tumor Phe-tRNA may represent activation of a new tRNA gene.

The plasmacytoma tRNA analysis suggests that tumors lack some of the biochemical flexibility that can be seen in normal organs; i.e. the ability to adapt the tRNA populations to the protein synthesis needs of the cell. This rigidity or fixed nature of tumor cells indicates that biochemical pathways may be fixed at time of malignant transformation. Conceivably this lack of adaptibility can be taken advantage of clinically or therapeutically.

Proposed Course:

The mRNA and cDNA preparations will continue, monitoring each preparation for purity in cell-free protein synthesis and RNA-DNA hybridization. Preparation of nuclear proteins will be expanded to include livers, spleens, and all plasmacytomas under study. Different fractionation schemes will be explored to separate the acidic nuclear proteins to be tested for DNA binding. The study of the effects of growing plasmacytomas and other tumors on liver cells will be continued to see if nonsecreting tumors also cause liver MUP levels to fall. IEF and RIA will test whether the decrease in liver MUP is selective for only certain of the 3 known MUP components.

Structure determination of tumor-specific Phe-tRNA will be continued by Dr. Grunberger and functional analysis of these Phe-tRNAs will be concluded in collaboration with Dr. Hatfield

Publications:

Mushinski, J.F., and Marini, M.S.: Multiple chromatographic peaks of phenylalanyl-tRNA associated with spontaneous hydrolysis of Y base during isolation. Biochim. et Biophys. Acta 476: 345-351, 1977.

ANNUAL REPORT OF THE METABOLISM BRANCH
SUMMARY OF SIGNIFICANT ACTIVITIES
NATIONAL CANCER INSTITUTE

October 1, 1977 through September 30, 1978

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with malignancy, in patients with immune deficiency diseases and a high incidence of neoplasia as well as in experimental animal systems to determine the nature of immune processes that are of importance in the surveillance against neoplastic cells and to define the nature of defects in the immunological system that result in an increased incidence of neoplasia. Major efforts in this area are also directed toward: 1) Defining events of cellular maturation, cellular interaction and cellular biosynthesis involved in the normal circulating antibody response. Specific emphasis is placed on studies of immunoregulatory cells including helper T cells, suppressor T cells and macrophages that regulate antibody responses and on studies of disorders of these immunoregulatory cell interactions in patients with immune dysfunction. 2) Identification of unique cell surface determinants on subpopulations of lymphoid cells with different functional capabilities. 3) The genetic control of the immune response especially as related to the immune response genes associated with the major histocompatability complex. 4) Studies of the mechanism of action of various cytotoxic cells and their contribution to host defense. 5) Detection of circulating tumor associated antigens. The second major goal of the Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic disease as well as those with non-neoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms and metabolic derrangements of biochemical control mechanisms are being investigated. Within this area a special emphasis is laid on the biochemical events accompanying normal growth and the hormonal control of this growth as it relates to our understanding of malignant growth.

Immunoregulatory Cell Interactions in Immune Response

A major effort of the Metabolism Branch over the past few years has been directed toward defining the major events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. These studies have placed special emphasis on defining the defects of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells that occur in patients with primary immunodeficiency diseases associated with a high incidence of malignancy, and in patients with malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then from B lymphocytes into immunoglobulin synthesizing plasma cells. The maturation of B cells into plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Specifically many antigens and mitogens require the presence of both helper lymphocytes of thymic origin (helper T cells) and macrophages as well as the B cells to induce a full antibody response. More recently it has been recognized that a separate class of thymic derived cells, suppressor T cells,may act as negative regulators of B cell maturation inhibiting this process. To study these events we have developed a series of techniques including techniques to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, techniques to assess helper T cell function, and to detect both increased and decreased functional activities of suppressor T cells. In the basic procedure peripheral blood lymphocytes are cultured with polyclonal activators of B cells *in vitro* and the immunoglobulin synthesized and secreted by these cells is quantitated using radioimmunoassay procedures. The first agent that we used in these precedures, pokeweed mitogen, is a prototype of a thymic and macrophage dependent activator of B cells. Over the past year a series of new activators of B cell immunoglobulin synthesis have been identified including *Nocardia opaca* extract, staphylococcal-phage-lysate, staphylococcal organisms of the Cowan's strain A and streptolysin O. These agents are useful since they vary markedly in terms of their requirements for T helper cells and macrophages and in terms of their capacity to activate suppressor T cells. A modification of the immunoglobulin biosynthesis procedure has been developed to assess the ability of macrophages to act as one of the helper cells required for B cell maturation and to detect activation of monocytes into cells that inhibit immunoglobulin synthesis. In addition an entirely new approach to the investigation of B cell differentiation has been developed which permits the precise quantitation of the numbers of lymphoid cells secreting each class of immunoglobulin. This technique, called the reverse hemolytic plaque assay, has been employed in studies of normals and patients with a variety of immunodeficiency, autoimmune and malignant disorders. Studies of peripheral blood of normal subjects demonstrated the presence of a lymphoid cell subpopulation actively secreting immunoglobulin with a mean of 189 lymphocytes secreting IgG, 182 secreting IgA and 121 secreting IgM per million circulating lymphoid cells examined were found.

Studies of the distribution of immunoglobulin secreting lymphocytes in various lymphoid organs showed 20 to 50 times more secreting cells per million lymphoid cells were present in the tonsils and bone marrow than in blood. In addition, cells secreting IgE could be detected in these lymphoid organs, but not in the blood. A series of studies also demonstrated that immunoglobulin secreting cells are a normal consituant of the lymphoid cells obtained by lavage of the lower bronchial alveolar tree of man. Such immunoglobulin secreting cells in the lung may play an important role in local pulmonary immune defense and the presence of IgE secreting cells in the lung may contribute to the particular sensitivity of this organ to allergic reactions.

This same assay was also employed in *in vitro* studies of B lymphoid cell differentiation. Kinetic studies of the presence of Ig secreting cells in cultures of blood lymphocytes stimulated with the polyclonal mitogen, pokeweed mitogen showed that on day three of culture an exponential rise in the number of immunoglobulin secreting cells began and the response peaked at 6-8 days of culture with approximately 15,000 immunoglobulin secreting cells being detectable. This response was shown to be dependent upon the interaction of T cells, B cells and macrophages in these cultures.

These techniques for studying immunoglobulin synthesis in vitro were used to determine whether homogenous populations of T cells from patients with T cell leukemias retain the capacity to help or suppress the synthesis of immunoglobulins from lymphocytes derived from normal individuals. It is hoped that the study of the monoclonal proliferations of malignant T cells and their products may prove as exciting in answering questions regarding cellular immunity and T cell regulation of humoral immune responses as myeloma cells and their paraproteins have proved to be in answering questions concerning humoral immunity. One of the most critical issues concerning the capacity of T cells to regulate human immunoglobulin synthesis is whether suppressor cell activity is generated by a single population of T cells acting alone or whether suppressor cell generation requires interaction between different populations of T cells neither of which has the capacity to affect suppression alone. The proof of such T-T interaction requires clear identification and separation of the two classes of cells involved. In human systems,this has been exceedingly difficult until now. We have taken advantage of an unusual patient with acute lymphoblastic leukemia and hypogammaglobulinemia prior to therapy to address this issue. In our studies we learned that the neoplastic T cells from this patient functioned as pro-suppressor cells. We observed that the patient's leukemic T-cells produced no detectable immunoglobulin and depressed the immunoglobulin production of co-cultured normal unseparated lymphocytes by 85-100% in the presence of pokeweed mitogen. However, suppression was observed only when cooperating normal T cells were present. Prior irradiation of either the leukemic cells or the cooperating normal cells nullified the suppressor effect. We, thus, were able to conclude that an interaction between at least two different T cell subsets is required for the generation of so-called suppressor-effector T cells in man. We extended these studies by analyzing whether normal T cells could secrete a soluble factor which could activate the leukemic cells under discussion to become suppressor effector cells. We learned that normal T cells when stimulated with pokeweed mitogen secrete a factor (or a series of factors) which may activate the leukemic cells to become direct suppressors without the further need of cooperating T cells in a pokeweed mitogen driven system of immunoglobulin production. These studies enabled us to construct a model for the induction of human suppressor effector cells. In this model, pro-suppressor T cells must interact with a different activating set of T cells before becoming fully functional suppressor T cells. The model developed from the use of these neoplastic T cells provide at least two testable expectations for further research. The first is that neoplastic diseases which represent proliferations at different steps of suppressor cell differentiation will be discovered. Second, patients with neoplasms comprised largely of suppressor effectors will be discovered, and these patients will have hypogammaglobulinemia directly related to the tumor cell burden. On the other hand, these patients who are discovered to have neoplasms of prosuppressor or suppressor activators will have hypogammaglobulinemia related to the residual number of functional capacity of non-neoplastic cooperating T cells as well as leukemic cells. To our knowledge, these studies represent the first proof of cooperative interaction between neoplastic and normal T cells in the generation of immunoregulatory effector T cells.

The nature of the defects leading to decreased immunoglobulin and antibody synthesis in patients with the primary immunodeficiency diseases was studied using pokeweed mitogen stimulated immunoglobulin synthesis assays and the reverse plaque assay discussed above. Patients with the diagnosis common variable hypogammaglobulinemia were shown to represent a variety of disorders with different fundamental defects at different steps in the maturation of bone marrow stem cells to plasma cell. Most patients had an intrinsic defect in the B cell plasma cell series. In some cases the patients could not produce B cells, in others B cells could not mature into plasma cells and in yet others plasma cells could synthesize but not secrete immunoglobulin. In contrast to the observation in normal individuals there were no lymphoid cells actively secreting immunoglobulins in the circulation of such patients. In addition the cells of such patients could not be induced by culture in vitro with pokeweed mitogen or other B cell activators to produce immunoglobulin molecules as assessed by the reverse hemolytic plaque assay or by radioimmunoassay of culture supernatants of immunoglobulin molecules.

A subgroup of patients with common variable hypogammaglobulinemia had an excessive number of activated suppressor T cells. Cocultures with cells from this subset of patients with agammaglobulinemia with normal cells resulted in strikingly diminished responses as the result of hyperactive suppressor T cells present in the blood of these patients. The kinetics of appearance immunoglobulin secreting cells in these cocultures showed that the exponential increase in secreting cells began normally but was aborted abruptly at between four and five days of culture. Since the suppressor cells must be present from the initiation of the cultures, these observations suggest that the suppressor T cells act on an early stage in B cell differentiation but that their effect does not become apparent until after the B cells have begun to divide and differentiate into immunoglobulin secreting cells.

A final group of patients with hypogammaglobulinemia were found to produce immunoglobulin normally in our culture systems despite profound defects in vivo. These patients appear to have a host environmental defect rather than an intrinsic defect of B or T cells. In a subgroup of these patients a suppressed response was observed when the patients own serum was added to the culture of either patient's cells or normal cells along with fetal calf serum. Thus we have demonstrated that a subset of patients with this agammaglobulinemia have a circulating humoral immunosuppressive factor which may account for their profound immunodeficiency.

Patients with ataxia-telangiectasia manifest cerebellar ataxia occulocutaneous telangiectasia, elevated alpha-fetoprotein levels, a defect of DNA repair following irradiation,a high incidence of neoplastic disease and a variable immunodeficiency state. The majority of patients have a small embryonic appearing thymus, have defects in cell-mediated immunity and markedly diminished levels of IgA and IgE. Two factors were shown to contribute to the low rates of IgA synthesis. The patients had an intrinsic defect of their IgA producing B cells and had a reduction of helper T cell activity. The patients' helper T cells did not cooperate normally with normal B cells for immunoglobulin synthesis in the pokeweed mitogen stimulated immunoglobulin system.

An extreme defect in helper T cells was shown to be the cause of the hypogammaglobulinemia in the form of severe combined immunodeficiency characterized by an absence of circulating T cells but normal numbers of B cells. The B cells of these patients could not synthesize immunogloublin molecules when cultured with pokeweed mitogen in vitro but synthesized immunoglobulin normally when normal T cells were added to the culture system. The primary defect in such patients might be a defect of T stem cells or to a failure of thymus function leading to a failure in the maturation of such stem cells into functional T cells.

Studies of the immunoregulatory role of T cells have been continued in an animal model of humoral immunodeficiency. In the chicken, a central lymphoid organ, the Bursa of Fabricus is the site of initial differentiation and development of B lymphocytes. If the Bursa is removed in the newly hatched chicken before B cells have emigrated to peripheral lymphoid organs, the chicken develops agammaglobulinemia. Lymphoid cells obtained from these agammaglobulinemia birds were found to exert a profound effect on the humoral immune system when they were transplanted into normal birds. Recipients of T cell grafts from agammaglobulinemia donors were found to have impaired antibody responses and to frequently develop agammaglobulinemia themselves. This transplantable or "infectious agammaglobulinemia" was found to be caused by suppressor T cells present in the transplanted cells from the original bursectomized donor. The immunodeficiency which developed in the recipient chickens was characterized by total absence of IgG, IgA, and IgM just as in the bursectomized donors, but differed from donor birds in that the recipient chickens had the persistence of B lymphocytes and germinal centers in their lymphoid tissues and continued to have a Bursa of Fabricus. Thus, these recipient chickens had developed an immunodeficiency state closely paralleling that seen in many patients with common variable hypogammaglobulinemia who also have persistence of B lymphocytes and evidence for excessive "suppressor T cell" activity.

Dysgammaglobulinemia in man is a disorder characterized by the deficiency of one or more classes of immunoglobulin with persistence of normal levels of the other immunoglobulins. When T cells from various agammaglobulinemic chickens were transplanted to normal recipients in limiting dilution assays, recipient birds were found to develop dysgammaglobulinemia with selective loss of only one class of immunoglobulin. The deficiency which ultimately developed in the recipient birds varied from donor to donor suggesting that suppressor cells for each individual class of immunoglobulin are present in the donors and that the predominant suppressor cell class is revealed by the limiting dilution assay. Antibody responses in the dysgammaglobulinemic birds were present in the non-suppressed Ig classes and as in the birds which developed agammaglobulinemia following transplantation, B cells continued to be present bearing the immunoglobulin of the suppressed Ig class. This Ig class specific suppression also closely parallels immune deficiency disorders seen in man and provides an excellent experimental model to further investigate the role of T cell regulation of Ig production and may allow the development of therapeutic approaches for the correction of such deficiencies in man.

In the previous studies the disordered suppressor cells was of the T cell series. Patients with multiple myeloma appear to have a different defect as the cause of their significant reduction in serum levels of polyclonal immunoglobulins and in the capacity to synthesize antibody in response to antigenic challenge. These patients have markedly reduced polyclonal immunoglobulin synthesis *in vitro*. In addition, in co-culture with normal cells they have a suppressor. However, in contrast to the observations in patients with common variable hypogammaglobulinemia or thymoma and hypogammaglobulinemia the cell appears to be a phagocytic mononuclear cell. Over the past year using an experimental model of transplantable myeloma in mice, we have also demonstrated that the hypogammaglobulinemia of such mice is due to development of a non-T cell phagocytic mononuclear cell. However, the transplantation of these myelomas to nude athymic mice was not associated with a marked reduction in immunoglobulin synthesis and did not lead to development of splenic suppressor cells observed in normal mice bearing these tumors. Thus it appears that although T cells are not the effectors of suppression they play a role in the chain of events leading to the development of such cells. The T cells may recognize antigens on the myeloma cells or their products and in turn may activate monocytes to become the suppressor cells that inhibit immunoglobulin synthesis.

Surface Determinants on Lymphocyte Subpopulations with Different Functions

A major advance in cellular immunology has followed the observation that T cells encompass subpopulations of lymphocytes with different and at times opposing functions. The T cells of mice with different functions bear different surface determinants. It is becoming widely accepted that human T cells with different functions may be characterized by their cell surface receptors for immunoglobulin molecules; that positive regulators of human immune responses (helper cells) express receptors for the Fc portion of IgM (referred to as Tμ cells) and that T cells which act as negative regulators of humoral immune responses (so-called suppressor cells) express receptors for the Fc portion of IgG (referred to as Tγ cells). We felt the concept that two distinct populations (Tμ and Tγ) with different immunoregulatory potential was so important that a considerable research effort on our part was appropriate. We learned that a rigid classification of T cells into so-called Tμ and Tγ cells is not appropriate. We found that, under the appropriate conditions, normal T cells may express Fc-IgM and Fc-IgG receptors simultaneously. In addition, there may be a transition of Tγ cells into Tμ cells. The signal for this transition appears to be an interaction with immune complexes. Furthermore, it is not possible to categorize Tγ cells as having exclusive suppressor function and Tμ cells as having exclusive helper function. Thus, under the proper conditions Tγ cells may exert a helper influence and Tμ cells may exert a suppressor influence. These studies may eventually help clarify the biologic role of Fc-IgM and IgG receptors of human lymphocytes. However, at the very least, they have already provided an important warning against the indiscriminate use of membrane Fc-IgG and Fc-IgM receptors to predict immunoregulatory function. Finally we have identified a receptor for IgA and one for IgM on normal B cells, chronic lymphocytic leukemia cells and B cell lines.

In other studies, we have developed evidence that human and mouse T cells possess an IgA-Fc specific binding site as well as the previously described receptors for IgG and IgM. The IgA-Fc receptor is demonstrated with a rosetting technique with ox-red blood cells coated with IgA myeloma proteins. The IgA binding sites thus demonstrated were shown by inhibition studies with IgA, IgG and IgM antigen-antibody complexes to be specific for IgA. In other studies it was shown that the binding site for IgA was the Fc component.

Genetic Control of the Immune Response

Discovery in the last decade of the existence of immune response (Ir) genes, which control the ability of an animal to respond to an antigen with a high degree of antigenic specificity, has opened the door to a potential understanding of the regulatory factors which control the immune surveillance against malignancy and infectious disease as well as the abnormal immune responses involved in the pathogenesis of autoimmune and other diseases. Most immune response genes studied have been those controlling the response to relatively simple synthetic antigens and hapten-carrier complexes. Over the past two years, we have studied the fine specificity of immune response gene control of the antibody response to the natural protein antigen, staphylococcal nuclease, and sperm whale myoglobin in mice. Antisera raised in different inbred strains of mice to these two natural protein antigens under H-2-linked immune response (Ir) gene control, have been studied for their specificity for different determinants on the antigen molecule. In the case of nuclease, H-2-linked immune response genes affect the proportion of antibodies to different determinants. The H-2 linkage of this pehnomenon has been confirmed in an F_2 study, and testing of additional strains suggests the existence of multiple genes in H-2 which have yet to be mapped. In the case of myoglobin, two immune response genes have been mapped to the I-A and I-C subregions of the H-2 complex. Recombinant strains with only the gene in I-C (Ir-Mb-2)produce an intermediate level of antibodies to whole myoglobin, lacking antibodies to the c-terminal region amino acid residues (132-153), whereas strains with only the gene Ir-Mb-1 in I-A make antibodies to this region. These results suggest that the responses to different determinants on the same antigen molecule are controlled independently by different genes, and that intermediate responsiveness is only the net effect of high responder genes for some determinants and low responder for other determinants.

A major example of a human disease wherein a disordered immune response is related to genes assocaited with the major histocompatibility complex as well as to genes on a separate chromosome. Studies directed toward understanding the pathogenesis and treatment of gluten sensitive enteropathy were continued. In previous studies we observed that approximately 80% of gluten sensitive enteropathy patients bear the HLA-B8 histocompatibility antigen. A similar incidence of this antigen was observed in dermatitis herpetiformis, a skin disease associated with gastrointestinal lesions essentially identical to those found in gluten sensitive enteropathy. During the present period we studied patients with dermatitis herpetiformis to determine the association of this disease with gluten sensitivity and gastrointestinal abnormalities. In particular we determined the relationship between the presence or absence of linear or granular IgA immunoglobulin deposits in the skin basement membrane zones and the presence of gastrointestinal disease. It was found that five patients with linear deposits in the skin did not have gastrointestinal abnormalities whereas five consecutive patients with granular deposits all had gastrointestinal abnormalities. Similarly patients with dermatitis herpetiformis and linear deposits did not have an elevated incidence of HLA-B8 whereas those with granular deposits had an elevated incidence of HLA-B8. On the basis of these findings it was apparent that there exists two forms of dermatitis herpetiformis, one associated with granular IgA deposits of the skin, gastrointestinal abnormalities and increase of HLA-B8 and one associated with linear IgA deposits in the skin, no GI abnormalities and a normal HLA-B8 incidence.

In yet other studies we examined the toxicity to patients with gluten sensitive enteropathy of an experimental bread which is lacking in alpha-gliadin. This bread was obtained from the United States Department of Agriculture with the use of chromosomally deficient grains. The bread was fed to five patients with gluten sensitive enteropathy and the patients were then evaluated for toxicity. It was found that in every patient studied the bread caused toxicity just as would be observed with normal bread. It was therefore concluded that the presence of alpha-gliadin in wheat is not necessary for development of toxicity in gluten-sensitive enteropathy.

Mechanism of Action of Cytotoxic Mononuclear Cells and Their Contribution to Host Defense.

Cytotoxic killer cells have been implicated in a number of systems of host defense including the destruction of malignant cells. The studies of the types of cells involved, the surface characteristics, specificity, and the mechanism of action of these various cytotoxic killer cells and their contribution to host defense were continued. Model systems have been developed for the study of killer T cells, cytotoxic K lymphocytes and cytotoxic monocytes in a series of distinct cytotoxic processes. These have included antibody dependent cellular cytotoxicity, mitogen induced cellular cytotoxicity, natural killer and monocyte dependent cytotoxicity.

Spontaneous or natural cell mediated cytotoxicity is a phenomenon of cell mediated killing which has been correlated with tumor resistance and which may play a significant effector role in immune surveillance. We have established assays of natural cytotoxicity for human cells employing Chang cell targets. Studies were directed primarily at defining the surface characteristics and specificity of cytotoxic effector cells in vitro. The human effector cells mediating "spontaneous" or "natural" cell mediated cytotoxicity were characterized as lacking cell surface complement receptors. These effector cells, like the effector cells mediating antibody-dependent cellular cytotoxicity may now be defined as non-adherent, non-phagocytic, surface immunoglobulin negative, B-cell alloantigen negative, complement receptor negative, Fc receptor bearing mononuclear cells. The identity of the spontaneous cell mediated cytotoxicity effector in experimental animals was clarified by demonstrating that in contrast to previous reports, this effector in guinea pigs possess Fc receptors. A different type of cell mediated killing, mitogen induced cellular cytotoxicity is a form of killing in which the immune effector cells are T cells that kill target cells in the presence of a plant mitogen or lectin. In humans this T-cell mediated mitogen-induced cellular cytotoxicity was not an exclusive functional capability of either the T-cell subset defined by surface receptors for IgG (Tγ) or that with IgM (Tμ) receptors. Studies of T-cell mediated cytotoxicity were extended to cell-mediated lympholysis reactions and trinitrophenyl-dependent cell mediated lympholysis reactions. With regard to cell mediated lympholysis in which cytotoxic lymphocytes are generated by in vitro immunization with allogeneic lymphocytes, the precursors of alloimmune cytotoxic lymphocytes were shown to be T-cells and precedures effectively enriching responder cell populations for Tμ cells enriched cytotoxic lymphocyte precursors. At the effector phase the functional cells were identified as T-cells which did not express either IgM or IgG-Fc receptors. Thus while both T-cell subsets possessed cytolytic activity in mitogen induced cellular cytotoxicity for the other cytotoxic systems studied the precursor was a Tμ cell while the effector was a T-cell which did not express Fc receptors.

Two additional models of non-specific cytotoxicity have been developed to study the role of rapidly acting cell mediated cytotoxicity in early host defense to foreign challenge. One model measures pure monocyte mediated killing. Development of cytotoxicity is due to an *in vitro* inactivation of a potent suppressor cell found in cells freshly derived from the animal. Interestingly several widely used stimulatory plant mitogens, especially pokeweed mitogen, act to dramatically stimulate cellular suppression of nonspecific monocyte mediated cytotoxicity. This model has lead to a new understanding of the cellular events involved in control of monocyte mediated cytotoxicity. A second model is dependent upon the presence of a non-antibody heat labile substance found in normal human plasma which can arm T cells as well as monocytes to kill.

Both of these models have been used to evaluate non-specific cytotoxic function using patients with severe immunologic disorders. This approach has been very fruitful. We have developed evidence that patients with common variable hypogammaglobulinemia, ataxia-telangiectasia and the Wiskott Aldrich syndrome have defective non-specific monocyte killing. Furthermore, we have evidence that each disease has a distinct defect.

Proline Biosynthesis and Metabolism

Proline, a nonessential amino acid, has a number of unique metabolic functions. Proline together with its hydroxylated derivative, 4-hydroxy-L-proline, constitute 20% of the amino acids in collagen. More importantly, the degradative produce of proline, Δ^1-pyrroline-5-carboxylate, is an intermediate linking the TCA and urea cycles. Thus, proline may have a regulatory role in intermediary metabolism.

Proline degradation - In studies using intact cultured cells, we showed that proline is converted to ornithine. The conversion is affected by the redox state of the cell. A high mitochondrial NADH/NAD ratio favors ornithine formation. Thus, the coupled reactions catalyzed by proline oxidase and ornithine aminotransferase can supply ornithine, a vital intermediate for both urea and polyamine synthesis. We also found that the enzyme, proline oxidase, is inhibited directly by fatty acyl-coenzyme A's. The effect is reversible, CoA derivatives rather than fatty acids are the effectors; chain length of the fatty acyl-CoA is critical with fatty acyl chain length greater than 12 carbons required for inhibition. We previously reported that lactate regulates proline oxidase activity by increasing the Km of the enzyme for proline. In contrast, fatty acyl-CoA's decrease the Vmax of the enzyme. In addition, we found differential sensitivities to fatty acyl-CoA's in various tissues. In brain mitochondria the Ki for fatty acyl-CoA is three-fold higher than in liver and kidney mitochondria. These findings may relate to the recognized metabolic changes accompanying the shift from amino acids to fats as a source of metabolic fuel. Our demonstration of regulation of proline oxidase supports our hypothesis that proline has a regulatory function in intermediary metabolism.

Proline synthesis - We previously showed that proline synthesized from ornithine is incorporated into proteins even when exogenous proline is available. To prove that this biosynthetic pathway for proline is physiologically important, we obtained mutant cells able to synthesize proline only from ornithine. Using these cells we showed that proline can be formed from ornithine at rates adequate to support maximal cell growth.

Mechanism of Action of Growth Hormone

An understanding of the biochemical events accompanying normal growth (as mediated by growth hormone, for example) may be a pre-requisite for understanding malignant growth. There is evidence that the anabolic action of growth hormone is mediated by a second protein designated somatomedin. A somatomedin-like polypeptide (MSA or multiplication stimulating activity) has been purified from the conditioned media of a rat liver cell line (BRL 3A) that multiplies continuously in tissue culture in serum free media. Several active species of MSA of different size have been purified and characterized as to relative activity in radioreceptor and bioassays.

A radioimmunoassay for MSA has been developed using rabbit antisera and homogeneous radioiodinated MSA tracer. The radioimmunoassay has been shown to be specific for MSA; other purified somatomedins including IGF-I and II, and somatomedin A compete with only 1% of the potency of MSA. Using this assay MSA has been detected in rat serum and found to be approximately three-fold lower in hypophysectomized (growth hormone deficient) rats than in normal rats.

Liver perfusion studies reported in the literature have supported the idea that MSA is made in the liver. In a collaborative study with Matthew Rechler (NIAMDD) and Howard Eisen (NICGID) it has been shown that a fetal rat liver organ culture system produces a peptide(s) identical or similar to MSA as determined by bioassay, radioreceptor assay and radioimmunoassay.

The pygmy mouse (pg/pg) has been proposed as a model of growth hormone resistance. We have found that somatomedin levels are normal in the pygmy mouse indicating that the genetic lesion in the growth hormone pathway must reside distal to somatomedin generation. In collaboration with Matthew Rechler (NIAMDD) the rat liver membrane somatomedin receptor has been found to have normal binding capacity and affinity for MSA.

Several recent reports have indivated that somatomedin purified from human plamsa have inhibited adenylate cyclase activity in vitro, consistant with the proposal of Pastan and others that in some cells in tissue culture lowered cyclic AMP levels are associated with more rapid growth. A study carried out in collaboration with Wayne Anderson (NCI) has shown that MSA inhibits prostaglandin stimulated increases in cyclic AMP levels and adenylate cyclase activity in chick embryo fibroblasts.

Porphyrin Metabolism

The efficacy of intravenously administered hematin in the therapy of acute intermittant porphyria was evaluated in 14 patients with acute attacks. The 14 patients were given 24 therapeutic courses with a course defined as approximately 3 mg/kg of hematin intravenously given daily for periods of 3-14 days. All patients responded with a decline of porphyrin precursor excretion (aminolevulinic acid [ALA] and porphobilinogen [PBG]), which could be decreased to normal levels by a sufficient dosage of hematin. Certain clinical manifestations, such as abdominal pain, tachycardia and hypertension improved considerably with hematin administration. As might be expected, established motor neuropathy was not significantly affected during the brief courses of hematin.

In one patient with regular, predictable, cyclic attacks of porphyria, beginning three days before her menstrual periods, hematin was shown to prevent these attacks when given once a week. This prophylactic effect was maintained for approximately six months.When hematin was stopped, the cyclic pattern of attacks recurred.

The total biochemical and clinical evidence thus far suggests that hematin is of value in the treatment of acute attacks of porphyria, particularly when given early in the attack. No side effects of hematin have been observed, other than occasional venous irritation.

In one patient with lead intoxication hematin caused a marked decrease of the excess δ-aminolevulinic acid (ALA) in the urine. It was assummed that hematin was acting on induced hepatic ALA synthetase. In animals with lead intoxication studies during the past year, hepatic ALA synthetase induction has not been demonstrable.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB- 04002 -09-MET

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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Metabolism Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were directed toward determining the steps involved in the differentiation of B cells into immunoglobulin synthesizing and secreting plasma cells. Special emphasis was laid on developing new techniques to study the role of helper T cells, macrophages and suppressor cells in these immune regulatory process and to define defects in these immunoregulatory cell interactions in patients with immune dysfunctions. Leukemias of both suppressor and helper T cells have been identified. Excessive numbers of suppressor T cells have been demonstrated in association with agammaglobulinemia and selective IgA deficiency. Excessive numbers of non-T cell suppressors have been demonstrated in multiple myeloma associated with immunodeficiency. Although T cells are not the effectors of suppression in multiple myeloma they are required elements in the chain of cells required for the generation of the suppressor monocytes.

Project Description:

Objectives: The objectives of the study were: to determine the sequential steps involved in the differentiation of lymphocyte B cells into immunoglobulin synthesizing and secreting cells. Major efforts were directed toward defining the role of suppressor T lymphocytes, helper T lymphocytes, and macrophages in this maturational processes. Disorders of these suppressor and helper interactions have been defined in the primary immunodeficiency and malignancy diseases of man. Overall the studies are directed at defining disorders of the host immunoregulation that lead to a high incidence of malignancy or that led to a high incidence of infection in patients who have malignancies of the T and B cell system. Finally studies were directed toward developing new techniques for the detection of circulating tumor-related antigens that could be used in cancer diagnosis or in monitoring the effectiveness of therapy.

Methods Employed: In vitro culture techniques of mitogen stimulated peripheral blood lymphocytes have been developed for the study of the terminal differentiation of B lymphocytes into immunoglobulin secreting cells. New techniques with B cells purified by immunoabsorbent columns and followed by removal of contaminating T cells by rosetting techniques have been used in co-culture studies to detect helper T cells in man. Co-culture studies have also been utilized for the detection of excessive numbers or reduced numbers of suppressor T lymphocytes. Lymphocytes depleted of adherent cells by a variety of techniques have been used to assess monocyte, helper and suppressor functions.

Major Findings: A major effort of the Metabolism Branch over the past few years has been directed toward defining the major events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. These studies have placed special emphasis on defining the defects of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells that occur in patients with primary immunodeficiency diseases associated with a high incidence of malignancy, in patients with autoimmune disorders as well as in patients with malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then from B lymphocytes into immunoglobulin synthesizing plasma cells. The maturation of B cells into plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Specifically many antigens and mitogens require the presence of both helper lymphocytes of thymic origin (helper T cells) and macrophages as well as the B cells to induce a full antibody response. More recently it has been recognized that a separate class of thymic derived cells, suppressor T cells, may act as negative regulatory of B cell maturation inhibiting this process. To study these events we have developed a series of techniques including techniques to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, techniques to assess helper T cell function and to detect both increased and decreased functional activities of suppressor T cells. In the basic procedure peripheral blood lymphocytes are cultured with polyclonal activators of B cells in vitro and the immunoglobulin synthesized and secreted by these cells is quantitated using radioimmunoassay procedures. The first agent that we used in these procedures, pokeweed mitogen, is a prototype of a thymic and macrophage dependent activator of B cells.

Over the past year a series of new activators of B cell immunoglbulin synthesis have been identified including Nocardia opaqua extract, staphylococcal phage lysate, staphylococcal organisms of the Cowan's strain A and streptolysin O. These agents are useful since they vary markedly in terms of their requirements for T helper cells and macrophages and in terms of their capacity to activate suppressor T cells. A modification of the immunoglobulin biosynthesis procedure has been developed to assess the ability of macrophages to act as one of the helper cells required for B cell maturation and to detect activation of monocytes into cells that inhibit immunoglobulin synthesis. The techniques for studying immunoglobulin synthesis in vitro were used to determine whether homogenous populations of T cells from patients with T cell leukemias retain the capacity to help or suppress the synthesis of immunoglobulins from lymphocytes derived from normal individuals. It is hoped that the study of the monoclonal proliferations of malignant T cells and their products may prove as exciting in answering questions regarding cellular immunity and T cell regulation of humoral immune responses as myeloma cells and their paraproteins have proved to be in answering questions concerning humoral immunity. The leukemic cells from the majority of patients with acute T cell leukemia did not function as helper or suppressor cells. However, we have recently studied functional neoplastic T cells from an unusual child with acute lymphoblastic leukemia and hypogammaglobulinemia that represent an exception to this generalization. We observed that following systemic chemotherapy this patient's immunoglobulin levels returned to normal during remission of the leukemia and dropped once again during leukemic relapse. The leukemic T cells from this child acted as potent suppressors of immunoglobulin synthesis when co-cultured in vitro with normal cells and the B cell activator pokeweed mitogen. One interesting feature of these leukemic cells was their apparent requirement for a cooperating subset of normal T cells before a full suppressor effect could be expressed. On the basis of this and other studies it was concluded that a T-T cell interaction between a pro-suppressor T cells and a suppressor activator T cell is required for the generation of suppressor effector cells. Through the use of cultured T cell supernatants it was shown that the leukemic cells were pro-suppressor cells with the normal T cell function as suppressor activators in the presence of pokeweed mitogen.

The leukemic T cells from patients with the Sézary syndrome were shown to function as helper T cells in some but not all cases. These cells could not be activated to become suppressor cells. Thus, in many cases the leukemic cells of patients with the Sézary syndrome represent a homogenous malignant expansion of helper T cells.

The nature of the defects leading to decreased immunoglobulin and antibody synthesis in patients with the primary immunodeficiency disease was studied using the immunoglobulin synthesis assay. Patients with the diagnosis common variable hypogammaglobulinemia were shown to represent a variety of disorders with different fundamental defects at different steps in the maturation of bone marrow stem cells to plasma cells. Most patients had an intrinsic defect in the B-cell-plasma cell series. In some cases the patients could not produce B cells, in others B cells could not mature into plasma cells and in yet others plasma cells could synthesize but not secrete immunoglobulin.

A subgroup of patients with common variable hypogammaglobulinemia had an excessive number of activated suppressor T cells. In some cases pokeweed mitogen was required in the system indicating that these patients had increased numbers of circulating non-activated pro-suppressor cells. In other cases small numbers of patients' cells suppressed the immunoglobulin synthesis of co-cultured normal cells stimulated by all polyclonal B cell activators studies. These patients appear to have increased numbers of activated suppressor cells.

A final group of patients with hypogammaglobulinemia were found to produce immunoglobulin normally in our culture systems despite profound defects _in vivo_. These patients appear to have a host environmental defect rather than an intrinsic defect of B or T cells. In a subgroup of these patients a suppressed response was observed when the patients' serum was added to the cultures of the patients' own cells or normal cells along with fetal calf serum. Thus we have demonstrated that a subset of patients with hypogammaglobulinemia have a circulating humoral immunosuppressive factor which may account for their profound immunodeficiency.

Patients with ataxia-telangiectasia manifest cerebellar ataxia occulocutaneous telangiectasia, elevated alpha-fetoprotein levels, a defect of DNA repair following irraidation, a high incidence of neoplastic disease and a variable immunodeficiency state. The majority of the patients have a small embryonic appearing thymus, have defects in cell-mediated immunity and markedly diminished levels of IgA and IgE. Two factors were shown to contribute to the low rates of IgA synthesis. The patients had an intrinsic defect of their IgA producing B cells and had a reduction of helper T cell activity. The patients' helper T cells did not cooperate normally with normal B cells for immunoglobulin synthesis in the pokeweed mitogen stimulated immunoglobulin system.

Severe combined immunodeficiency diseases encompasses a series of different diseases defined by gross functional impairment of both humoral and cell-mediated immunity that is usually congenital and frequently genetic. Affected individuals have rampant infections with a whole rnage of organisms.

An extreme defect in helper T cells was shown to be the cause of the hypogammaglobulinemia in the form of severe combined immunodeficiency characterized by an absence of circulating T cells but normal numbers of B cells. The B cells of these patients could not synthesize immunoglobulin molecules when cultured with pokeweed mitogen _in vitro_ but synthesized immunoglobulin when normal T cells were added to the culture system. The primary defect in such patients might be a defect of T stem cells or might be due to a failure of thymus function leading to a failure in the maturation of T stem cells into functional T cells.

In the studies discussed above the disordered suppressor cells were of the T cell series. Patients with multiple myeloma appear to have a different defect as the cause of their significant reduction in serum levels of polyclonal immunoglobulins and in the capacity to synthesize antibody in response to antigenic challenge. These patients have markedly reduced polyclonal immunoglobulin synthesis in vitro. In addition, in co-culture with normal cells they have a suppressor. However, in contrast to the observations in patients with common variable hypogammaglobulinemia or thymoma and hypogammaglobulinemia the cell appears to be a phagocytic mononuclear cell. Over the past year using an experimental model of transplantable myeloma in mice, we have also demonstrated that the hypogammaglobulinemia of such mice is due to development of a non-T cell phagocytic mononuclear cell. However, the transplantation of these myelomas to nude athymic mice was not associated with a marked reduction in immunoglobulin synthesis and did not lead to development of splenic suppressor cells observed in normal mice bearing these tumors. Thus it appears that although T cells are not the effectors of suppression they play a role in the chain of events leading to the development of such cells. The T cells may recognize antigens on the myeloma cells or their products and in turn may activate monocytes to become the suppressor cells that inhibit immunoglobulin synthesis.

Significance to Biomedical Research: The techniques developed for the study of the terminal differentiation of B cells and for helper and suppressor T cells is of the same value in the study of B cell maturation as is the lymphocyte blastogenic response to PHA in the study of thymus-dependent lymphocyte (T cell) maturation. Thus, in vitro techniques are now available to evaluate both arms of specific immune response in man. One can now study the physiological factors regulating this response, one can define defects in the process in patients with immunodeficiency disorders or neoplasia of the B cell or T cell lymphocyte system. In addition, such studies can be used to determine the effect of chemotherapeutic or other agents of the terminal differentiation of B cells in parallel with studies of the effects of these agents on T cells so that one can define agents which preferentially affect T cell immunity and those which preferentially effect B cell immunity.

Proposed Course: Studies directed toward understanding the pathways and controlling mechanisms of the synthesis, transport and catabolism of serum physiological and pathophysiological factors controlling the sequential development of B cells into immunoglobulin synthesizing cells. Special emphasis will also be placed on the development of new techniques for the detection of circulating tumor-related antigens. These latter studies will include the evaluation of the synthetic rates of catabolism of such tumor-related antigens.

Honors and Awards

3rd Lucy Klein Memorial Lecture, Northwestern University
8th Henry M. Stratton Lecture, American Society of Hematology

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB-04003-09-MET

PERIOD COVERED
October 30, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald P. Tschudy, M.D.	Senior Investigator	MET	NCI
OTHER:	Joel M. Lamon, M.D.	Clinical Associate	MET	NCI
	Abraham Atsmon, M.D., Ph.D.	Visiting Scientist	MET	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objectives are to study the mechanisms controlling heme biosynthesis and biochemical and clinical aspects of the porphyrias. Further work will attempt to apply the knowledge gained from the above objectives to the study of tumors and tumor-host relationships and other conditions which effect porphyrin metabolism as well as primary diseases of porphyrin metabolism.

Project Description:

Objectives: To study the mechanisms controlling heme biosynthesis and biochemical and clinical aspects of the porphyrias. To apply the knowledge gained from the above objectives to the study of tumors and tumor-bearing hosts as well as primary diseases of porphyrin metabolism.

Methods Employed: Quantitative enzyme determinations, chemical determination of porphyrins and porphyrin precursors, isolation, purification and measurement of tissue heme, use of tissue preparations and isotope methods in studying metabolic pathways and computer and mathematical analysis of certain mathematical models.

Major Findings: 1. The study of the effect of intravenously administered hematin in acute intermittent and variegate porphyria has involved 242 doses in 14 patients, given as 24 courses. A course is defined as approximately 3 mg/kg of hematin intravenously given daily for periods of 3-14 days. All patients responded with a decline of porphyrin precursor excretion (aminolevulinic acid {ALA} and porphobilinogen {PBG}), which can be decreased to normal levels by a sufficient dosage of hematin. Certain clinical manifestations, such as abdominal pain, tachycardia and hypertension improved considerably with hematin administration. As might be expected, established motor neuropathy was not significantly affected during the brief courses of hematin.

In one patient with regular, predictable, cyclic attacks of porphyria, beginning 3 days before her menstrual periods, hematin was shown to prevent these attacks when given once a week. This prophylactic effect was maintained for approximately 6 months. When hematin was stopped, the cyclic pattern of attacks recurred.

The total biochemical and clinical evidence thus far suggest that hematin is of value in the treatment of acute attacks of porphyria, particularly when given early in the attack. No side effects of hematin have been observed, other than occasional venous irritation.

2. Hematin has been administered to two patients with erythropoietic protoporphyria. In the first patient, who had a greatly increased level of fecal propoporphyrin, hematin caused a pronounced decline of this level. In the second patient, who had only a slight elevation of fecal protoporphyrin, no significant effect of hematin on this level was seen. However, both patients developed small areas of skin lesions which clinically and pathologically resembled areas of allergic vasculities. This phenomenon was not seen in any of the patients with acute intermittent or variegate porphyria.

3. A method has been developed for the preparation of rat liver cell suspensions which are free of heme and hemoglobin. These cells and chick embryo liver cells are being studied for the effect of various compounds on the heme content of the cell. Allylisoprophylacetamide (AIA), a potent

porphyrinogenic compound, causes a decline of liver cell heme, followed by an increase of heme above control levels. The heme content of liver cells has been found to be very sensitive to environmental conditions.

4. In one patient with lead intoxication, hematin caused a marked decrease of the excess S-aminolevulinic acid (ALA) in the urine. It was assumed that hematin was acting on induced hepatic ALA synthetase. In animals with lead intoxication studies during the past year, hepatic ALA synthetase induction has not been demonstrable.

5. The hormonal preparation, Danazol, has produced exacerbations of acute porphyria.

Significance to Biomedical Research: These studies are helping to elucidate some of the fundamental chemical defects of diseases which involve alterations of porphyrin metabolism. New forms of treatment have been developed for some of these diseases as a result of these studies.

Proposed Course: (A) To continue studies of the factors controlling heme and heme-enzyme synthesis in normal and pathologic tissues. (B) To continue studies of biochemical abnormalities of porphyria in man and experimental animals. (C) To study control mechanisms of heme synthesis in tumors and livers of tumor-bearing hosts.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

NCI-Z01-CB-04004-09-MET

PERIOD COVERED

October 30, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of Amino Acid Biosynthesis and Degradation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James M. Phang, M.D.	Senior Investigator	MET	NCI
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CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An important factor in cellular growth and metabolism is the biosynthesis degradation, and transport of amino acids. This project emphasizes the regulatory interplay in amino acid metabolism.

Project Description:

Objectives: The biosynthesis, degradation and transport of amino acids play an important role in cellular growth and metabolism. Not only are amino acids the necessary building blocks for proteins but also amino acids may play important roles in intermediary metabolism, e.g. alanine, ornithine. This project emphasizes the biosynthesis and degradation of proline because the metabolic intermediates of proline metabolism provide an important link between the TCA and urea cycles. The mechanisms regulating proline biosynthesis and degradation in normal and malignant cells are poorly understood. These studies may elucidate changes in growth rates, energy allocation and protein synthetic rates in tumors. The project is relevant to objective 3, Approaches 1 and 5 of the National Cancer Program.

Methods Employed: We are primarily using animal cells maintained in long-term tissue culture. Cells can be cloned to insure a genetically homogeneous population. Mutants are isolated by standard techniques. A variety of biochemical techniques have been utilized to study amino acid biosynthesis and degradation in cultured cells. Uptake of amino acids may be assessed by using labeled L-amino acids and rates of protein and collagen synthesis can be obtained by incubating cells with L-proline-C^{14} and separation of the radioisotopically incorporated amino acids as proline and hydroxyproline by ion exchange column chromatography. Specific metabolic rates of amino acids can be determined by isolating labeled products from labeled precursors. Methods have been developed to recover ornithine from proline and glucose from proline and alanine. The enzymes for biosynthesis and degradation of proline are assayed by using appropriate labeled precursors and isolating the products by ion exchange chromatography. Our methods are sensitive enough to measure these enzymes in leucocytes isolated from 10 ml of blood. Biochemical studies on specific enzymes include the isolation of mitochondria, solubilization of enzymes from mitochondrial particles and purification of enzymes.

Major Findings: In mammalian cells the committed step of proline biosynthesis is catalyzed by enzyme Δ^1-pyrroline-5-carboxylate reductase which reduces Δ^1-pyrroline-5-carboxylate (PC) to proline. PC, on the other hand, is the product of the reaction catalyzed by ornithine aminotransferase. PC also may be produced from glutamic acid although the enzymatic steps remain undefined in mammalian cells. The proline degradative pathway includes oxidation of proline by PC by proline oxidase and conversion of PC to glutamate by PC dehydrogenase.

We have shown that proline degradation plays an important role in intermediary metabolism. Not only can glucose be formed from proline as shown by recovery of labeled carbons in glucose from labeled proline, but proline also augments the conversion of labeled alanine to glucose.

The effect of proline on glucogenesis from alanine is specific in that glutamate, ornithine or other amino acids cannot augment the conversion of alanine to glucose. We also found in cultured LLC-RK1 cells (rabbit-kidney cells) that ornithine can be formed in significant amounts from proline. Using labeled proline precursors, we recovered labeled ornithine by Dowex column chromatography. The production of ornithine is stimulated by rotenone, which blocks NADH oxidase and increases the mitochondrial NADH/NAD ratio. Glycolytic intermediates e.g. pyruvate and lactate, inhibit the formation of ornithine from proline. In kinetics studies of ornithine formation the apparent Km for proline was 2.5mM which approximates the Km of partially purified proline oxidase for substrate proline. Thus we showed that proline oxidase and ornithine aminotransferase can be functionally coupled in intact cells to produce significant amounts of ornithine, a vital intermediate in polyamine synthesis and urea formation.

We previously showed that proline oxidase is induced by glucocorticoids and is regulated directly by lactate. We now have shown that long-chain fatty acyl coenzyme A's are regulators of proline oxidase activity. In both mitochondrial and solubilized enzyme preparations, we found that fatty acyl-CoA's but not fatty acids inhibited proline oxidase. The Ki for palmitoyl-CoA was 5-10 μM. Although both saturated and unsaturated fatty acyl-CoA's were effective, chain length of the fatty acid moiety was critical. Fatty acyl chain length greater than 12 carbons was required for inhibition. The effect was reversible and specific. Kinetics studies showed that the inhibitory effect was due to a decrease in enzyme Vmax which contrasts with the lactate-mediated increase in Km for proline. These findings may relate to the recognized metabolic changes accompanying the shift from amino acids to fats as a source of metabolic fuel. Tissue differences were observed in the sensitivity of proline oxidase to palmitoyl-CoA. Proline oxidase from brain mitochondria was much less sensitive to palmitoy-CoA than enzymes from liver or kidney mitochondria. Ki for palmitoyl CoA was 5 μM in the hepatic enzyme. In contrast the Ki of the brain enzyme was 15 μM. This difference in proline oxidase from brain and liver may be involved in the recognized ability of brain to oxidize fatty acids. The above findings support our thesis that proline oxidase is regulated to furnish intermediates for the TCA and urea cycles.

We have continued our studies of the biosynthesis of proline. Previously we showed that proline synthesized from ornithine is incorporated into proteins even when exogenous proline is available. To prove that this biosynthetic pathway for proline is physiologically important, we isolated revertant cells from proline auxotrophs (CHO-K1) which have either the glutamate pathway CHO(ORN-, GLU+) or the ornithine pathway CHO(ORN+, GLU-). By comparing the nutritional requirements for these prototrophic revertants with the parent proline auxotrophic cell line, CHO(ORN-, GLU-), we found that CHO(ORN-, GLU-) cells required proline or PC for growth: CHO(ORN-, GLU+) cells grew at optimal rates in MEM without proline. CHO(ORN+, GLU-) cells grew well without proline but required ornithine. These studies prove that proline endogenously synthesized from either ornithine or glutamate is adequate for optimal growth rates.

Significance to Biomedical Research: The regulation of amino acids for protein synthesis may play an important role in controlling cell growth. The enzymes involved in amino acid biosynthesis and degradation are affected by genetic, nutritional and hormonal factors. Our studies on the proline metabolic enzymes are contributing to the understanding of these factors. Specifically, genetic defects in the proline metabolic enzymes have elucidated some of the physiologic derangements secondary to a metabolic block. Elucidation of the molecular mechanisms for regulating proline biosynthesis and degradation will lead to an understanding of biochemical regulation in mammalian cells. Furthermore, our findings that (1) proline degradation supplies metabolic intermediates, (2) affects and is affected by the energy state of the cells and (3) is closely regulated all suggest that proline may play an important role in cellular intermediary metabolism.

Proposed Course: The evidence suggests that the end point of the proline biosynthetic and degradative enzymes is not only to provide proline for the cell but also to regulate a spectrum of reactions in intermediary metabolism. The regulation may involve anaplerosis, redox states and metabolic energy. Because proline oxidation is directly coupled to electron transport, ATP may be produced directly especially when NADH oxidase is rate-limiting. Furthermore, because PC can be either oxidized or reduced in NADH or NAD dependent reactions, the redox state of the cell may be affected. We are emphasizing studies to prove these regulatory functions using a variety of experimental approaches. Several cell lines with defined enzyme defects are available. These include fibroblasts from patients with inherited metabolic disorders as well as cell lines isolated and enzymatically defined by our laboratory. The Pro/Re mice, an inbred strain with deficient proline oxidase, also will be included in these studies. Specific questions will include whether proline or PC and the related metabolic enzymes influence NADH/NAD ratios, urea synthesis and glucose formations. We are also studying a human breast tumor cell line (MCF-7's) and defining proline metabolism in these cells as compared to normal cells.

Biochemical studies will emphasize two enzymes, proline oxidase, and PC reductase. The regulation of proline oxidase by lactate and palmitoyl-CoA will be explored using purified proteins. Direct ligand-protein interactions will be sought and the characteristics of the interaction defined. Preliminary studies on PC reductase have shown that the hepatic enzyme is activated with heat and dialysis. Furthermore the interaction with NADH suggests that NADH-dependent subunit interactions may be involved in the activation.

Publications:

Kowaloff, E.M., Granger, A.S. and Phang, J.M.: Glucocorticord control of hepatic proline oxidase. Metabolism 26:893-901, 1977.

Downing, S.J., Phang, J.M., Kowaldoff, E.M., Valle, D. and Smith, R.J.: Proline oxidase in cultured mammaline cells. J. Cell. Physiol. 91:369-376, 1977.

Smith, R.J., Downing, S.J. and Phang, J.M.: Enzymatic synthesis and purification of L-pyrroline-5-carboxylic acid. Anal. Biochem. 82:170-176, 1977.

Kowaloff, E.M., Phang, J.M., Granger, A.S. and Downing, S.J.: Regulation of proline oxidase activity by lactate. Proc. Natl. Acad. Sci. USA 74:5368-5371, 1977.

Kowaloff, E.M., Phang, J.M., Granger, A.S. and Downing, S.J.: Glucocorticoid induction of proline oxidase in LLC-RK1 cells. J. Cell. Physiol. (In press).

Smith, R.J. and Phang, J.M.: Proline metabolism in cartilage: The importance of proline biosynthesis. Metabolism. (In press).

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PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
NCI-Z01-CB-04014-09-MET

PERIOD COVERED October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of the Immune Response in Normal and Pathological States

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were directed toward the analysis of immune response in normal humans and patients with a variety of immune disorders, as well as analysis of humoral and cellular immune responses of experimental animals.

Project Description:

Objectives: Studies were directed toward the analysis of the humoral and cellular immune responses of experimental animals with particular emphasis on the study of experimental models of human disease. Studies were directed toward the analysis of the immune responses of normal humans as well as individuals having a variety of immune disorders with concentration on immunodeficiency disorders as well as immunologic disorders of the gastrointestinal tract. These studies included the investigation of patients with gluten-sensitive enteropathy, dermatitis herpetiformis, inflammatory bowel disease, as well as patients with ataxia telangiectasia, common variable hypogammaglobulinemia, and intestinal lymphangiectasia. Each of these diseases are associated with a high incidence of reticuloendothelial malignancy and the mechanism underlying the association between malignancy and these diseases was sought.

Methods Employed: A host of techniques were used to assess immunologic function of experimental animals and patients with disorders of the immune system. Circulating B cells in peripheral blood and in tissue were enumerated with carefully prepared fluorescein-conjugated F(ab')2 antiserum with specificity for heavy chains of immunoglobulins. Circulating T cells were enumerated with sheep red blood cell rosette techniques. Antibody responses were assessed by microhemagglutination techniques and in vitro proliferative responses were assessed with tritiated thymidine incorporation following exposure to specific and nonspecific mitogens. Mitogen-induced cytotoxic effector cells as well as antibody dependent and natural cytotoxic effector cells were assessed with ^{51}Cr-release assays using red cell and nucleated metabolically active target cells. Homogenous cell populations were isolated with the use of Sephadex anti-Fab columns, rosetting techniques and the use of colloidal iron followed by passage through a magnetic field. Short term organ culture was used in conjunction with solid phase immunoabsorption techniques to measure mucosal immunoglobulins; long term organ culture techniques were used to study in vitro models of intestinal disease. Cells obtained from various tissues as well as from peripheral blood were cultured in vitro under a variety of conditions. Supernatants from these cell cultures were assayed by radioimmunoassay in order to measure immunoglobulin cells produced in culture. Patients with immunologic disorders were studied with metabolic turnover techniques to measure immunoglobulin synthesis and catabolism.

Major Findings:

Studies on the Regulation of the IgA Immunoglobulin Response - During this period a broad study of the regulation of IgA immunoglobulin synthesis was brought to fruition. Specifically, we studied the role of T cells in the regulation of IgA responses and showed that IgA responses were governed by subsets of T cells which are distinct from those regulating IgM responses. The system used involves in vitro lipopolysaccharide (LPS)-driven immunoglobulin synthesis of murine lymphocytes wherein the immunoglobulin produced is measured by specific double antibody radioimmunoassays.

In initial studies LPS-driven immunoglobulin synthesis was determined in four different mouse tissues: peripheral lymph node, spleen mesenteric lymph node and Peyer's patches. IgM was synthesized by all four tissues whereas IgA was synthesized by spleen, mesenteric lymph node and Peyer's patches, but not be peripheral lymph nodes. The radio of IgA to IgM and the absolute amount of IgA synthesized was always highest in Peyer's patch cultures. Since it is possible that differences in the distribution of regulatory T cells may explain the variation in IgA biosynthesis in these different tissues, the effects of T cell activation by Concanavalin A (Con A) on the LPS-driven immunoglobulin synthesis was explored. In such studies, it was found that the addition of Con A to the culture medium resulted in a significant depression of IgM synthesis in culture of peripheral lymph nodes, spleen, mesenteric lymph node and Peyer's patches. In contrast, the addition of Con A to cultures had a quite different effect on IgA synthesis: although there was suppression of IgA in the spleen there was little change in the mesenteric lymph node and a marked enhancement of IgA synthesis in the Peyer's patches. To further define the effect of Con A on T cell activation, immunoglobulin synthesis in cultures of unfractionated cells to which purified Con A activated T cells were added. In these studies T cells were obtained by nylon column fractionation and pulsed with Con A for 40 hours; the pulsed T cells were then added to fresh spleen and Peyer's patch cells and the resulting mixutres of Con A-pulsed T cells and indicator B cells were cultured seven days in the presence of LPS; finally, the supernatants were assayed for immunoglobulin content by radioimmunoassay. It was found that both Con A-pulsed spleen T cells and Con A-pulsed Peyer's patch T cells suppressed spleen IgM B cells; in addition, both Con A-pulsed spleen T cells and Con A-pulsed Peyer's patch T cells suppressed Peyer's patch IgM B cells. This result confirms the earlier results obtained when Con A was simply added directly to cell cultures and indicates that the T cell regulation of IgM was the same in both spleen and in the Peyer's patch. The effects of Con A-pulsed T cells in IgA synthesis were quite different from those on IgM biosynthesis. In this case Con A-pulsed spleen T cells caused suppression of IgA synthesis by indicator spleen or Peyer's patch B cells whereas Con A-pulsed Peyer's patch T cells cause enhancement of IgA synthesis by indicator spleen and Peyer's patch B cells. This again confirms the original experiments wherein Con A was simply added directly to cell cultures and is consistent with the concept that, as far as IgA synthesis is concerned, the Peyer's patch T cellpopulations contained an increased ratio of IgA helper to suppressor activity compared to spleen T cells.

A further inference that may be drawn from the above data is that the IgA and IgM T cell regulatory systems are separate. To substantiate this point, we asked whether the ratio of helper to suppressor T cell activity for IgM was also greater in Peyer's patches than in spleen but that this increased ratio was masked in the former organ by an increased sensitivity of IgM B cells to suppressor T cell signals. To answer this question the regulation of IgM synthesis by varying numbers of spleen or Peyer's patch Con A-pulsed T cells was compared. It was found that the addition of Con A-pulsed Peyer's patch T cells induce even greater suppression of IgM synthesis than Con A-pulsed spleen T cells over a range of T cell/B cell ratios. Thus, there is no evidence of increased T cell helper activity for IgM in the Peyer's patch and if anything the IgM suppressor activity is increased in that organ. From these experiments it may, therefore, be concluded that when the two tissues, Peyer's patches and spleen are compared for regulatory activity of IgM and IgA synthesis, the regulation in the spleen favores IgM suppression as well as IgA suppression whereas the regulation in the Peyer's patches favores IgM suppression and IgA enhancement. This independent variation of T cell regulation of IgM and IgA allows one to conclude that separate T cell regulatory systems exist for IgA and IgM.

Having thus established that IgA biosynthesis is under the control of a separate T cell regulatory system it was possible to analyze changes in the magnitude of IgA biosynthesis which normally occurs as an animal ages, with the view of assessing the role of regulatory T cells on this phenomenon. In these experiments young mice (6 to 8 weeks of age) were compared to adult mice (8 months of age) as to their capacity to produce IgM and IgA immunoglobulin in vitro. With respect to IgM biosynthesis in spleen, mesenteric lymph node, and Peyer's patchesn, no major differences between the different age age groups was found. On the contrary, when IgA synthesis was examined, the two age groups differed in that the 8 week old animals had little or no IgA synthesis in spleen or mesenteric node, with significant amounts of IgA synthesis in Peyer's patches whereas the 8 month old group was able to synthesize IgA in all three tissues. These in vitro studies corresponded to serum IgA levels in the two age groups wherein it was found that young animals have very low serum IgA levels and adult animals have high IgA levels. It was thus, apparent that IgA synthesis in peripheral tissues such as spleen and mesenteric lymph node and the occurrence of high serum IgA levels was age-dependent.

To study the role of regulatory T cells on this age-dependent change, studies using tissues of old and young animals were performed using Con A as an activator of helper and suppressor activity. In such studies it was found that the addition of Con A to cultures of tissues of all ages causes suppression of IgM synthesis, an effect that could also be shown with Con A-pulsed cells. On the other hand, the addition of Con A to cultures of young animals' cells (or of Con A pulsed cells) led to suppression of IgA synthesis in all tissues studied (including Peyer's patches) whereas the addition of Con A to cultures of old animals' cells led to suppression of IgA synthesis in the spleen and mesenteric lymph nodes but to enhancement of IgA synthesis in the Peyer's patch. In other studies mixture experiments were performed in which old and young T and B cells of various tissues were co-cultured in an effort to directly demonstrate that changes in IgA biosynthesis occur in association with changes in net T cell regulatory activity. In these studies it was shown that T cells obtained from spleen and Peyer's patches of older animals resulted in more IgA synthesis than similar cells obtained from young animals. Thus, the young animal has IgA biosynthesis limited to the Peyer's patch, Con A-inducible suppression of IgA in the Peyer's patch and low serum IgA levels whereas the older animals have IgA synthesis in all lymphoid tissues, Con A-inducible help for IgA in the patch and a serum IgA that is considerably higher than in the young animal. From these studies it seems apparent that the change in the magnitude of IgA synthesis occurring with age is dependent, at least in part, on IgA specific T cell regulatory activity.

The above finding could be due to obligate maturational events in T regulatory cells or may be due to exposure to environmental antigens and mitogens on regulatory cells. To decide between these two possibilities, germ-free mice were studied before and after conventionalization. It was found that the adult germ-free mice before conventionalization showed a pattern similar to young conventional mice in that IgA synthesis was present mainly in Peyer's patch that Con A caused suppression of Peyer's patch IgA synthesis. Following conventionalization, a sharp rise in IgA synthesis occurred between the first and second week; moreover during this period there was a shift away from Con A-inducible suppression and toward Con A-inducible help. Thus, the regulatory T cells important to IgA synthesis appear to be under the influence of antigenic and/or mitogenic influences present in the environment.

In summary, these studies show that at least one factor in the development of the mature IgA system is dependent on the IgA-specific T cell regulatory system. With the devleopment of this system IgA responses occur in the Peyer's patches which are then peripheralized so that IgA producing B cells appear in other lymphoid tissues. This development appears to depend, at least in part, on environmental influences.

IgA Receptors of Murine Lymphoid Cells - In an effort to more directly identify the immunoregulatory cells important to IgA biosynthesis, receptors specific for IgA on lymphoid cells were sought in various organs. In the past it had been shown that receptors on lymphoid cells capable of binding immunoglobulin through the Fc component had been identified for both IgG and IgM. In these studies it was established that IgA also binds to lymphoid cells through its Fc receptor. The method used in these studies to identify IgA binding sites involved a rosetting assay in which chemically modified ox red blood cells (ORBC) were coated with IgA to form an indicator cell. These indicator cells were then mixed with lymphoid cell populations and rosettes formed by indicator cells bound to lymphocytes sought under phase microscopy. In each case the source of IgA used to coat the ORBC's was a mouse myeloma ascites fluid containing IgA antibody having specificity for a determinant on the modified ORBC's. For instance in the case of MOPC-315 IgA the ORBC was modified with TNP and in the case of SAPC-10 IgA the ORBC was modified with arabinogalactan.

It was found that 4-6% of mouse spleen cells form rosettes with IgA coated RBC's (IgA rosettes). Equivalent numbers of IgA rosettes were obtained when several different myeloma ascites fluid were used as a source of the IgA bound to appropriately modified ORBC's or indeed when purified MOPC-315 IgA was used as a source of the IgA bound to ORBC's. However, spleen cells exposed to uncoated modified ORBC's or modified ORBC's pre-exposed to irrelevant myeloma ascites fluid did not form appreciable numbers. The IgA binding sites became more apparent following incubation of the cells for 24 hours at 37°C. Presumably binding sites were occupied in vivo by bound IgA and became available for assay following the incubation procedure.

To establish the specificity of the receptors on lymphoid cells binding IgA, inhibition studies with IgA and IgG antigen-antibody complexes as well as purified immunogloublins were carried. To detect inhibition, spleen cells were incubated with immune complexes prior to mixing with coated indicated ORBC's. Multiple inhibition studies using coated ORBC's indicated that IgA antigen-antibody complexes invariably caused a 50% or greater inhibition of IgA rosettes whereas IgG complexes caused no inhibition of IgA rosettes. To determine if IgG and IgM binding sites were present on separate cells, rosette studies were performed on whole spleen cell populations as well as populations depletion of IgG binding cells. In these studies, IgG rosettes were formed and the cells subjected to Ficoll-Hypaque separation in order to remove IgG binding cells. This process was repeated and the residual population was mixed with IgG or IgA-coated ORBC's to determine the number of IgG and IgA binding cells left following the cell depletion steps. It was found that the depletion procedures resulted in a 60-80% reduction of IgG binding cells but at most a 20% reduction of IgA binding cells. Thus, we conclude that most cells bearing IgA binding sites do not concomittantly bear IgG binding sites.

To determine if IgA is binding to cells in these assays through its Fc determinants F(ab')2 fragments of IgA were prepared. It was found that equimolecular concentrations of purified IgA derived from MOPC-315 ascites and F(ab')2 fragments of the same purified IgA were quite different in their capacity to inhibit rosette formation. F(ab')2 fragments of IgA were not able to inhibit the formation of IgA rosettes whereas purified IgA was able to cause such inhibition. Finally, to define the cell type binding IgA, T cell-enriched cell populations were prepared by nylon column passage of host spleen cells with rabbit anti-T antiserum. It was found that both cell populations contained roughly equal percentages of IgA binding cells and it seemed likely that IgA binding sites were found both on B cells and on T cells. This concept is supported by the finding that studies of IgA binding cells of peripheral lymph nodes disclose that this tissue yielded cells which bound IgA but not IgG. Since any K cells present in peripheral lymph node tissue would bind IgG, the presence of IgA binding cells and the absence of IgG binding cells in peripheral lymph nodes indicates that the IgA is binding to either B cells and T cells or more likely both types of cells rather than to K cells.

The finding that IgA can bind to lymphoid cells through the Fc receptor suggests that the IgA T cell regulatory system shown in studies discussed above may in fact be composed of cells bearing IgA Fc receptors. These studies lead to the possibility that specific immunoglobulin class specific regulatory T cells may be directly demonstrated.

Gluten-Sensitive Enteropathy - Studies directed toward understanding the pathogenesis and treatment of gluten-sensitive enteropathy (GSE) were continued. GSE is a disease of the GI tract associated with malabsorption, villous flattening, inflammatory cell infiltration of the lamina propria and an increased incidence of neoplasia. It is due to the toxic effect of wheat proteins (gluten) on the gastrointestinal mucosa. In previous studies we observed that approximately 80% of the GSE patients bear the HLA-B8 histocompatability antigen. A similar incidence of this antigen was observed in dermatitis herpetiformis (DH), a skin disease associated with gastrointestinal lesions essentially identical to that found in GSE.

In current studies performed in collaboration with Dr. S.I. Katz and Dr. T. Lawley of the Dermatology Branch, NCI, we studied the incidence of villous atrophy in dermatitis herpetiformis. In this regard it is known that patients with DH may have either linear or granulat IgA immunoglobulin deposits in the skin basement membrane zones. We, therefore, performed jejunal biopsies of patients with either linear or granular deposits to determine which patients had GI abnormalities (villous atrophy) and which patients did not have GI abnormalities. It was found that of five patients with linear deposits in the skin, none had GI abnormalities whereas of five patients with granular deposits, all had GI abnormalities. Patients with DH with linear and granular deposits could also be differentiated by HLA typing; the patients with the granular abnormalities had a high incidence of HLA-B8 whereas those with linear abnormalities had a normal incidence of HLA-B8. On the basis of these findings it is apparent that there exists two forms of DH: one associated with granualr IgA deposits in the skin, GI abnormalities and an increased incidence of HLA-B8 and one associated with linear IgA deposits in the skin, no GI abnormalities and a normal HLA-B8 incidence.

In other studies we determined that toxicity in patients with GSE in experimental bread which is lacking in alpha-fliadin. This bread was obtained from the United States Department of Agriculture from Dr. D.D. Kasards and his colleagues and is dervied from a genetic strain of wheat which is lacking in the chromosome material necessary for the synthesis of alpha-gliadin. Other gliadins, however, such as beta-gliadin or gamma-gliadin are still synthesized by this wheat. The bread so attained was fed to some five patients with GSE. The patients were studied for changes in absorption and loss of architecture following the bread ingestion. It was found that all patients suffered a deterioation in the structure and function of the GI tract after 10-14 days of ingestion of the genetically deficient bread. These studies are consistent with the hypothesis that alpha-gliadin is not necessary for the reduction of toxicity in GSE and toxicity may be generated by other gliadins.

The studies with the experimental bread are part of an on-going study of the nature of the toxic components in wheat which are necessary for the causation of GSE. In this connection we are currently testing chemically defined fragments of alpha-fliadin in organ culture studies in an attempt to more specifically identify the toxic fractions.

Significance to Biomedical Research: Gluten-sensitive enteropathy is an important disease model in which the role of histocompatability antigens in the control of immune response can be studied in humans. Our research in this areas had two goald: 1) identification of specific immunochemical determinants in wheat proteins specifically involved in disease pathogenesis; and 2) determinants on immunologically relevant cells. With formed regard we have used in vitro organ culture techniques in order to identify toxic wheat protein components; in latter regard we have identified that particular HLA linked and non-HLA linked cell surface antigens are related to gluten-sensitive enteropathy. Studies on the precise mechanism of how these receptors function to elicit hyper-responsiveness to gluten protein and gluten-sensitive enteropathy will shed light on the entire process of genetic control of antigen responsiveness in humans. This in turn can be expected to clarify pathogenetic mechanisms in autoimmune diseases, immunodeficiency diseases, and neoplastic diseases.

In our related studies of the regulation of the immune response on mucosal surfaces we have established that particular T cell subclasses may exert control over the immune response occurring at mucosal areas. Additionally, we have accumulated evidence that this regulation is at least in part responsible for the changes in IgA synthesis seen by exposure to environmental antigens in the gastrointestinal tract. It is reasonable to suppose that abnormalities in the control (regulatory) system identified here may be responsible for disease states. For instance, gluten-sensitive enteropahty, as mentioned above, may be due to abnormal regulatory control of the immune response to gluten protein, in this case mediated through particular genetically controlled receptors on the cell surfaces. In a similar vein, other gastrointestinal diseases may involve a defect in the control of IgA synthesis at mucosal sites. In particular, the inflammatory bowel diseases may be characterized by abnormal local regulatory mechansism leading to viral invasion and subsequent inflammation in the gastrointestinal tract. Immunodeficiency diseases, specifically involving the IgA immune system may also be due to faulty regulatory control. In this connection, IgA deficiency may represent a cell defect in the particular IgA specific T cell system involved in the control of IgA biosynthesis.

Proposed Course: Patients with gluten sensitive enteropathy will continue to be studies in order to unravel the fole of HLA antigens in the pathogenesis of disease. An in vitro stimulatory system will be sought to study the interaction of gluten protein with lymphoid cells in patients with gluten sensitive enteropathy. In this regard, several promising systems have been preliminarily tested. These include: 1) the in vitro production of polyclonal immunoglobulin in response to exposure to gliadin protein fractions; in studies so far conducted gliadin has been shown to stimulate IgA immunoglobulin production by lymphocytes obtained from GSE patients but not by similar cells obtained from normals; 2) the in vitro stimulation of cells by gliadin bound to cell surface membranes; this approach can utilize either immunoglobulin synthesis or tritiated thymidine uptake as a measure of stimulation; gliadin bound to syngeneic cells may be a more appropriate stimulator of lymphoid cells because of recent evidence gathered in this laboratory that cell recognition in the effector limb of immune response of gluten-sensitive enteropathy probably involves recognition of histocompatability antigens. Having established one or more specific stimulating systems, the effect of relevant antibody on the inhibition or further stimulation of the cells will be evaluated. Antibodies of relevance are antibodies to particular HLA specificities (either for B or D-locus antigens) or maternal antibodies with specificity for certain B cell antigens. In these studies an attempt will be made to alter the stimulation of cells by gliadin by preincubating cells with the appropriate antisera or with appropriate antisera in the presence of complement in an attempt to either block the relevant receptors or to lyse relevant cells bearing receptors. In this study we hope to establish a link between HLA antigens or B cell antigens in the initiation of the immune response in gluten-sensitive enteropathy.

In yet other studies we propose to continue attempts to demonstrate binding purified fractions of gluten proteins to both lymphoid cells and gut epithelial cells. In this connection we also hope to demonstrate gliadin binding of skin to DH patients since it is likely that such binding accounts for the skin lesions encountered in these diseases. Attempts during the past year to demonstrate such binding have been unsuccessful; however, these binding studies have utilized purified gliadin components which may not be specifically related to the binding process. Additional materials will be tested which constitute breakdown products of gliadin protein.

Studies of the regulation of the immune response will proceed in several directions. Firstly, an attempt to identify the IgA-specific T regulatory cell will be made. In this regard, T cell bearing Fc receptors are prime candidated inasmuch as cells bearing Fc receptors in other systems have been shown to play regulatory roles. Accordingly, purified T cell populations will be depleted of IgA receptors by rosetting with IgA-coated ox red blood cells. The resulting population of T cells will then be added to indicator B cell populations in the presence of lypopolysaccharide and the capacity of such B cell populations to support IgA responses will be determined. In other studies IgA-Fc receptor cells will themselves be isolated and added back to appropriate indicator systems. The effects of these cell manipulations on IgG and IgM synthesis will also be noted. Since it has been shown that IgA synthesis is totally T cell dependent even in the presence of B cell mitogens, such studies can be expected to test for the presence of IgA helper activity in IgA Fc-bearing cells. In other studies, measurements of suppressor activity in IgA Fc-IgA cells will be evaluated with the use of concanavalin A stimulated cells.

Secondly, the role of IgA regulatory T cells will be assessed in a variety of physiological situations. For instance, different mouse strains will be analyzed for the capacity to synthesize IgA and such capacity we will correlate it with IgA regulatory T cell fucntion. In other studies the effect of different drugs in IgA synthesis will be assessed and then analyzed from the point of view of effect of these drugs on T cell regulatory function which is specific for IgA. Thirdly, in preliminary studies we have shown that Kupfer cells bear IgA Fc receptors. Such receptors are also present to a lesser extent on peritoneal macrophages. It is, therefore, of great interest to determine the role of such receptors on macrophages on the control of IgA responses. This will be studied with in vitro antibody production utilizing Michell-Dutton antibody stimulation in vitro. Fourthly, and finally, humoral factors necessary for IgA production will be sought. We currently feel that the role of T regulatory cells is to expand T cell clones which have been pre-programmed to produce IgA immunoglobulin. Such pre-programming is under the influence of factors produced locally in the mucosal system; consequently using the test systems already developed, we plan to study the effect of extracts derived from mucosal epithelial cellsin an attempt to show that such extracts contain which can pre-program cells to produce IgA.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB-04015-09-MET

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of Development and Function of Humoral and Cellular Immune Mechanisms of Host Defense

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Michael Blaese, M.D.	Senior Investigator	M NCI
OTHER:	Andrew V. Muchmore, M.D.	Senior Investigator	M NCI
	E. Clinton Lawrence, M.D.	Clinical Associate	M NCI
	David G. Poplack, M.D.	Senior Investigator	PO NCI
	David L. Nelson, M.D.	Senior Investigator	M NCI
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COOPERATING UNITS (if any)

Pediatric Oncology Branch, DCT, NCI
Arthritis Branch, NIAMDD

LAB/BRANCH

Metabolism Branch

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study is directed toward determining mechanisms important to the development and function of humoral and cellular immune responses; the nature of deficiency in immune function; and developing approaches to prophylaxis and/or therapy of infections and neoplastic disease associated with immune processes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The objectives of this study were to determine mechanisms important to the development and function of both humoral and cellular immune response in animals and man; to determine the nature of the deficiency in immune function characterizing such disease states as hypogammaglobulinemia, ataxia telangiectasia, the Wiskott-Aldrich syndrome, intestinal lymphangiectasia, and advanced malignancy; and, to develop approaches to prophylaxis and/or therapy of the infectious and neoplastic disease frequently associated with defective immune processes. This work is directly related to Objective 2, Approach 1 of the National Cancer Plan.

Methods Employed: Antibody responses of experimental animals and man were determined after immunization with bacterial vaccines such as brucella abortus, S. typhosa, and pertussia; with particulate cellular antigens such as sheep, burro, and chicken RBC's; with purified protein antigens such as KLH, diphtheria and tetanus toxoids, guinea pig-γ-globulin and bovine-γ-globulin; with purified polysaccharide antigens such as pneumococcal polysaccharides types I, II, III, and the V_i antigen of E. coli. The antibodies were detected by standard techniques or by newly developed micro methods of bacterial agglutination, red cell hemolysis, and passive hemagglutination using $CrCl_3$ as an agent to couple the antigens to a carrier RBC surface. In addition, modifications of the Farr antigen binding assay were used.

Cellular immune responses were measured by delayed hypersensitivity skin tests, contact sensitivity to DNCB, and skin allografts. Lymphocyte transformation in vitro was carried out utilizing non-specific mitogens such as PHA, PWM and staph filtrate; specific antigens such as SLO, SKSD, diphtheria and tetanus toxoids, KLH and candida albicans; and allogeneic cells in mixed leukocyte culture. Lymphocyte function in vitro was also measured by production of mediators of cellular immunity such as lymphotoxin, MIF, and lymphocyte derived chemotactic factor for monocytes (LDCF) and by cytotoxicity reactions with antibody sensitized target cells or with non-specific mitogen activated effector lymphocytes. Immunoglobulin secretion by activated lymphocytes was measured by a newly developed reverse hemolytic plaque assay.

Studies of the characteristics of various cell surface receptors on immunocompetent lymphocytes and monocytes-macrophages utilized auto-radiography, immunofluorescence microscopy, and cellular rosette formation.

Major Findings: Mechanisms of immunoregulation of the interactions of immunocompetent cells has been a major interest of the Cellular Immunology Section for several years and a variety of techniques and model systems have been developed for these studies. Recently an entirely new approach to the investigation of B cell differentiation has been developed which permits the precise quantitation of the numbers of lymphoid cells secreting each class of immunoglobulin. This technique, called the reverse hemolytic plaque assay (RHPA), has been employed in studies of normals and patients with a variety of immunodeficiency, autoimmune and malignant disorders.

Initially, studies of peripheral blood demonstrated the presence of a lymphoid cell subpopulation actively secreting immunoglobulin in normal subjects. A mean of 189 lymphocytes/million cells were found to secrete IgG. 182 IgA secreting lymphocytes (IgA-SL) and 121 IgM-SL/million lymphoid cells were also found. Studies of patients with agammaglobulinemia revealed a lack of such IgSL for any Ig class in the blood while patients with selective IgA deficiency were found to lack IgA secreting cells while having normal numbers of cells secreting IgG and IgM. Significantly increased numbers of IgG and IgA secreting cells were found in the blood of patients with active systemic lupus erythemotisus and the increase in IgG secreting cells in this disorder was shown to have the highest degree coorelation with disease activity of any laboratory test for SLE including anti-DNA antibodies.

Studies of the distribution of IgSL in various lymphoid organs showed 20 to 50 times more IgSL/million lymphoid cells were present in the tonsils and bone marrow than in blood. In addition, cells secreting IgE could be detected in these lymphoid organs, but not in the blood. A series of studies also demonstrated that IgSL are a normal constituant of the lymphoid cells obtained by lavage of the lower bronchial alveolar tree of man. Such immunoglobulin secreting cells in the lung may play an important role in local pulmonary immune defense and the presence of IgE secreting cells in the lumg may contribute to the particular sensitivity of this organ to allergic reactions.

This same assay was also employed in in vitro studies of B lymphoid cell differentiation. Kinetic studies of the presence of Ig secreting cells in cultures of blood lymphocytes stimulated with the polyclonal mitogen pokeweed mitogen showed that on day 3 of culture an exponential rise in the number of IgSL began and the response peaked at 6-8 days of culture with approximately 15,000 IgSL being detectable. This response was shown to be dependent upon the interaction of T cells, B cells and macrophages in these cultures. Cells from patients with agammaglobulinemia failed to respond to such cultures. Co-cultures of normal cells with cells from a subset of patients with agammagloublinemia resulted in strikingly diminished responses as the result of hyperactive suppressor T cells present in the blood of these patients. The kinetics of IgSL appearance in these co-cultures showed that the exponential increase in IgSL began normally but was aborted abruptly between days 4 and 5 of culture. Since the suppressor cells must be present from the initiation of cultures, these observations suggest that the suppressor T cells act on an early stage in B cell differentiation but that their effect does not become apparent until after the B cells have begun to divide and differentiate into immunoglobulin secreting cells. Still another group of patients with agammaglobulinemia were found to produce immunoglobulin normally in our culture system despite their profound defect in vivo. These cultures were performed in fetal calf serum supplemented media and when the patients own serum was added to the cultures a suppressed response was observed. Thus, we have demonstrated that a subset of patients with this agammaglobulinemia have a circulating humoral immunosuppressive factor which may account for their profound immunodeficiency.

Studies of the immunoregulatory role of T cells have been continued in an animal model of humoral immunodeficiency. In the chicken, a central lymphoid organ, the Bursa of Fabricus is the site of initial differentiation and development of B lymphocytes. If the Bursa is removed in newly hatched chicken before B cells have emigrated to peripheral lymphoid organs, the chicken develops agammaglobulinemia. This immune deficiency state is characterized by total absence of B lymphocytes, immunoglobulins in the serum and secretions are undetectable, and all lymphoid tissues lack their characteristic components associated with the system of humoral immunity. T lymphocyte differentiation and function is not deficient in these bursectomized agammaglobulinemic chickens. However, T cells obtained from these birds were found to exert a profound effect on the humoral immune system when they were transplanted into normal birds. Recipients of T cell grafts from agammaglobulinemic donors were found to have impaired antibody responses and to frequently develop agammaglobulinemia themselves. This transplantable or "infectious agammaglobulinemia" was found to be caused by suppressor T cells present in the transplanted cells from the original bursectomized donor. The immunodeficiency which developed in the recipient chickens was characterized by total absence of IgG, IgA, and IgM just as in the bursectomized donors, but differed from donor birds in that the recipient chickens had the persistence of B lymphocytes and germinal centers in their lymphoid tissues and continued to have a Bursa of Fabricus. Thus, these recipient chickens had developed an immunodeficiency state closely paralleling that seen in many patients with common variable hypogammaglobulinemia who also have persistence of B lymphocytes and evidence of excessive "suppressor T cell" activity.

Further study of these birds with "infectious agammaglobulinemia" demonstrated that the suppressor T cells develop in the donor birds after 10 weeks of age and are not present in detectable quantities before that time. Immunoglobulin production in the recipient birds ceased almost immediately after injection with the suppressor cell population suggesting that these cells may be exerting an effect directly on the immunoglobulin producing plasma cells. B lymphocytes are initially present in normal numbers in the recipient birds but eventually fall to low numbers further suggesting that the suppressor cell may also act directly on B cells or their precursors.

Dysgammaglobulinemia in man is a disorder characterized by the deficiency of one or more classes of immunoglobulin with persistence of normal levels of the other immunoglobulins. When T cells from various agammaglobulinemic chickens were transplanted to normal recipients in limiting dilution assays, recipient birds were found to develop dysgammaglobulinemia with selective loss of only one class of immunoglobulin. The deficiency which ultimately developed in the recipient birds varied from donor to donor suggesting that suppressor cells for each individual class of immunoglobulin are present in the donors and that the predominant suppressor cell class is revealed by the limiting dilution assay. Antibody responses in the dysgammaglobulinemic birds were present in the non-suppressed Ig classes and as in the birds which developed agammaglobulinemia following transplantation, B cells continued to be present bearing the immunoglobulin of the suppressed Ig class. This Ig class specific suppression also closely parallels immune deficiency disorders seen in man and provides an excellent experimental model to further investigate the role of T cell regulation of Ig production and may allow the development of therapeutic approaches for the correction of such deficiencies in man.

Studies of the mechanism of action of various cytotoxic cells and their contribution to host defense were continued. Model systems have been developed for the study of killer cells in two separate and distinct cytotoxic processes. Antibody dependent cellular cytotoxicity (ADCC) is a form of cell mediated killing in which non-immune effector cells attack and kill target cells sensitized with IgG antibody. Mitogen induced cellular cytotoxicity (MICC) is a form of cell mediated killing in which non-immune effector cells kill target cells in the presence of a mitogen or lectin. Both MICC and ADCC are surface mediated killing events which do not require phagocytosis of the target cell. The mechanism of the surface mediated cytotoxicity has been obscure. Inhibitors of effector cell DNA, RNA and protein synthesis have not in general inhibited the cytotoxic capacity of these cells. Quabain, a cardiac glycoside, functions as an inhibitor of Na, K and Ca^{++} membrane transport systems. Addition of quabain to MICC and ADCC reactions inhibited MICC by greater than 95% while inhibiting ADCC to a much less significant degree. EDTA and EGTA, also inhibited MICC reactions to a significantly greater extent than ADCC. The inhibitory effects of these agents on MICC reactions could be completely reversed by the addition of Ca^{++} to the cell cultures. The differential effect of Quabain and Ca^{++} chelation on MICC and ADCC was shown to be the result of the preferential action of these inhibitors on the cytotoxic function of certain classes of effector cells. In MICC, the cytotoxic action of monocytes, PMN's, T lymphocytes, and K lymphocytes are all inhibited by ouabain while in ADCC, only monocyte mediated killer activity is inhibited while K cell mediated cytotoxicity is preserved. These data demonstrate a critical role for Ca^{++} ion flux in the cytotoxic action of effector cell populations involved in MICC and clearly demonstrate different biochemical requirements for cells mediating ADCC. These observations provide the first evidence of heterogeneity in the mechanisms by which cytotoxic cells exert their killer function and may provide an approach for altering the function of these cells in certain pathological conditions.

Evaluation of the immune capacity of patients with the rare syndrome of familial lymphohistiocytosis were performed in collaboration with Drs. Ladisch and Poplack of P.O.B., NCI. This disorder is characterized by infiltration of lymphoid tissues with masses of normal appearing histiocytes and is associated with hepatosplenomegally, erythrophagocytosis, hemolysis and consumption coagulopathy, recurrent fevers, wasting and increased susceptibility to infections. Immunologically the patients were found to be anergic to all recall skin test antigens. In vitro evidence of abnormal cellular immune function included depressed lymphocyte proliferative responses to specific antigens and imparied monocyte mediated cytotoxic function. Plasma from these patients suppressed the responses of normal lymphocytes. Investigation of the suppressive plasma revealed hyperlipidemia with triglyceride levels between 180-960 mg% (normal upper limit 150 mg%) and the degree of in vitro immunosuppression correlated with the extent of the hyperlipidemia. Further investigation of the immunosuppressive effects of plasma with hyperlipidemia showed that patients with a variety of disorders of lipid metabolism have in vitro and occasionally in vivo evidence of defective cellular immune function. Preliminary studies suggest that the immunosuppressive material in the plasma is associated with LDL and VLDL and may be GM ganglioside. This is a particularly intriguing observation since studies in mice have demonstrated that certain T cell associated antigens may bind to GM ganglioside and that this result in the activation of suppressor T cells.

Significance to Biomedical Research: The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune system in animals and man. They indicate that disorders of immune function may be related to the absence or malfunction of such central lymphoid organs as the thymus or Bursa of Fabricus or to immaturity of such auxillary systems as the macrophage required for the processing of antigens in the initiation of an immune response. In addition, they indicate that immunoregulatory cells and humoral materials may have a profound effect on the function of the immune system both in health and disease. These studies have also provided new insights into some basic defects in accessory immune elements which provide new avenues for work designed to correct malfunctions in immunity in malignant and immunodeficiency disorders.

Proposed Course: Continuing studies directed toward understanding the normal pathways involved in the development and function of the immune system and application of this new knowledge in approaches to the prevention and therapy of malignant disease.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB-04016-09-MET

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanism of the Anabolic Action of Growth Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Peter Nissley, M.D.	Senior Investigator	MET	NCI
OTHER:	Alan C. Moses, M.D.	Expert	MET	NCI
	Robert White, Jr., M.D.	Clinical Associates	MET	NCI

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Lab of Molecular Biology, NCI

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A radiommunoassay for multiplication stimulation activity (MSA) has been developed. Using this RiA, MSA has been measured in rat serum and found to be three-fold lower in hypophysectomized (growth hormone deficient) rats than in normal rats. MSA or a similar polypeptide has been shown to be produced in a fetal rat liver organ culture system by bioassay, radioreceptor assay, and radioimmunoassay. MSA has been shown to inhibit prostaglandin stimulated increases in cyclic AMP levels and adequate ayclase activity in chick embryo fibroblasts. A liver membrane preparation from the pygmy mouse exhibited normal binding capacity and binding affinity for MSA.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The understanding of normal growth may be a prerequisite to understanding malignant growth. One experimental model for the study of normal growth has been the growth hormone treated hypophysectomized rat. It has been proposed that the anabolic action of growth hormone is mediated by a second circulating protein designated somatomedin. The object of this project is to purify somatomedin, develop in vitro tissue culture systems responsive to somatomedin and study the sequence of biochemical events accompanying growth.

Methods Employed: Somatomedin (MSA or multiplication stimulating activity) is being purified from the conditioned medium of rat liver cells in culture using ion exchange chromatography, gel filtration and preparative disc acrylamide electrophoresis. Serum starved cells in culture are being utilized to study the action of MSA. DNA synthesis is measured by incorporation of treated thymidine into DNA. Cell surface receptor radioimmunoassay and serum somatomedin binding protein studies utilize iodinated MSA as radioligand.

Major Findings: A radioimmunoassay for MSA has been developed using rabbit antisera and homogenous MSA tracer. Human somatomedins (IGS I and II, and somatomedin A) compete for MSA tracer binding to antibody but at less than 1% the potency of MSA. This radioimmunoassay has been utilized to measure MSA in rat serum. Serum from hypophysectomized rats has 3-fold lower concentrations of MSA than found in normal serum.

The pygmy mouse has been proposed as a model of growth hormone resistance. We have found that somatomedin levels are normal in the pygmy mouse, thus the genetic lesion in the pygmy mouse must be distal to somatomedin generating at the level of the somatomedin receptor or beyond. Matthew Rechler's laboratory has evaluated the somatomedin receptor on rat liver membranes of the pygmy mouse and found it to be indistinguishable from normal with respect to capacity and affinity.

Liver perfusion studies have been reported that support the idea that somatomedin is generated in the liver. In collaboration with Matthew Rechler and Howard Eisen it has been shown that fetal rat liver in organ culture produces MSA by criteria of bioassay, radioreceptor assay and radioimmunoassay.

In collaboration with Wayne Anderson it was shown that MSA causes a reduction in PGE stimulated cyclic AMP accumulation in both NRK^1 cells and chick embryo fibroblasts (CEF). MSA also was effective in inhibiting both PGE_1 and flouride stimulated activities of CEF adenylate cyclase of crude membrane preparation.

Significance to Biomedical Research: The understanding of the biochemistry and regulation of normal growth should provide a framework for understanding the abnormalities of growth regulation in malignancy. The elucidation of the growth peptides required for the growth of cells in tissue culture contribute to the eventual establishment of completely defined media for cells in tissue culture. Studies of the interaction of growth peptides with cell surface receptors define early steps in hormone action and may help in the elucidation of how this protein-membrane interaction leads to an anabolic reponse. Specifically, this research supports objective 3, approaches 1 and 2 and objective 4, approach 1 of the National Cancer Plan.

Proposed Course: MSA will be purified in sufficient quantities for amino acid sequenching to be carried out in the laboratory of Darrell Liu (Bureau of Biologics).

The MSA radioiuumnoassay will be utilized for further measurement in rats. Studies will include a survey of MSA levels in organs, further serum measurements to establish hormonal regulation of MSA and measurement in fetal blood and organs.

Growth hormone deficient mutant mice and hypophysectomized rats will be treated with MSA to see whether or not MSA can reproduce anabolic effects of growth hormone *in vivo*.

A clinical protocol is being conducted that is designed to address the question of whether or not the serum binding protein for somatomedin is under growth hormone control.

Ten patients with growth hormone deficiency are being treated with growth hormone. Serum obtained before and after growth hormone treatment is being utilized to measure the size and amount of the somatomedin binding protein.

In collaboration with Matthew Rechler and Howard Eisen, the fetal rat liver organ culture system will be utilized to study hormonal control of MSA production.

Publications:

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Moses, A.C., Nissley, S.P., Rechler, M.M., Short, P.A., and Podskalny, J.M.: The purification and characterization of multiplication stimulating activity (MSA) from media conditioned by a rat liver cell line. International Symposium on Somatomedins and Growth, Santa Margherita Ligure (Genoa, Italy) March 1-3, 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB- 04017-09-MET

PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biology of the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Stephen Shaw, M.D.	Clinical Associate	IMM	NCI
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COOPERATING UNITS (if any)

LAB/BRANCH Metabolism Branch

SECTION Immunophysiology

INSTITUTE AND LOCATION NIH, NCI, Bethesda, Maryland 20014

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER:

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☑ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☑ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were undertaken to investigate the immunologic responsiveness of normal individuals and patients with immunologic disorders as well as experimental animals serving as models of human diseases.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Studies were undertaken to investigate the biology of immunologic responsiveness in normal individuals as well as patients with a variety of primary and secondary immunodeficiency diseases and disorders suspected of being the result of immunologically-mediated processes. Animal models of particular relevance to human diseases were also studied. Insight was sougth into the mechanisms underlying perturbations of immunologic function present in these patients particularly those defects leading to an increased incidence of neoplasia.

Methods Employed: Emphasis was placed on defining the morphological and functional characteristics of various leukocyte and lymphocyte populations. For these studies, separation procedures based on the presence of certain surface receptors on only sub-populations of cells were employed to generate highly purified leukocytes and lymphocyte sub-populations. Techniques empolyed consisted of fractionation of cells over insolubilized immunoabsorbents including Sephadex G-200 anti-Fab, plastic-adherent anti-Fab, and plastic adherent antigen-antibody complexes. Other cell separation techniques employed erythrocytes coated with various proteins, which, following interaction with cells bearing receptors for the specific proteins result in dense erythrocyte-lymphoid aggregates (rosettes) that may be separated from non-rosetting cells by buoyant density centrifugation. These separation techniques have been utilized in series and in parallel to yield highly purified sub-populations of cells for structural and functional analysis.

Morphologic analysis of mononuclear leukocyte sub-populations have included enumeration of macrophages by latex phagocytosis assays and staining for non-specific esterase. Bone marrow derived lymphocytes (B-cells) were enumerated by direct and indirect immunofluorescent staining for several cell surface characteristics including surface immunoglobulin (sIg), receptors for the Fc portion of complexed immunoglobulin (Fc receptors) and genetically controlled alloantigens (B-cell alloantigens-Ia alloantigens). B-cells were also enumerated by rosetting techniques employing IgM antibody and complement coated erythrocytes (complement receptors). Thymus-derived lymphocytes (T-cells) were enumerated by their receptors for sheep erythrocytes. T-cells were further separated into sub-populations on the basis of Fc receptors for IgM ($T\mu$) or IgG ($T\gamma$). Additional sub-populations of cells (K-cells) were defined on the lack of receptors characteristic of macrophages, B-cells and T-cells and the presence of high avidity Fc receptors.

The functional analysis of isolated leukocyte and lymphocyte sub-populations was primarily directed toward the definition of the cytotoxic effector potential of these cells. Use was made of a variety of target cell types in assays of "natural" or "spontaneous" cell mediated cytotoxicity (SCMC), mitogen-induced cellular cytotoxicity (MICC), antibody-dependent cellular cytotoxicity (ADCC), cell-mediated lympholysis (CML), and TNP-dependent cell-mediated lympholysis (TDCML).

Major Findings: Cytotoxic Effector Potential of Isolated Leukocyte and Lymphocyte Sub-Populations - Studies were continued to define the surface characteristics of cytotoxic repertoir of several isolated leukocyte and lymphocyte sub-populations. In previous human studies the effector cell(s) mediating SCMC and ADCC were shown to be present in a sub-population of non-adherent, sIg negative, B-cell (DRW) alloantigen negative, Fc-IgG receptor positive lymphocytes. In additional studies the effector cell(s) mediating SCMC and ADCC were shown to lack complement receptors which are present on macrophages and B-cells. Thus the effector cell(s) in human peripheral blood mediating SCMC and ADCC were further defined as being complement receptor negative. Although it is now well accepted that the effector cell(s) mediating SCMC in humans posses Fc-IgG receptors, considerable controversy continues to exist regarding the presence of Fc receptors on SCMC effectors in experimental animals. In order to clarify the identity of the SCMC effector in experimental animals, the SCMC effector activity of sub-populations of lymphocytes obtained from guinea pigs was ascertained. Using cell separation techniques similar to those employed in humans, the guinea pig splenic SCMC effector was shown by both positive and negative selection techniques to be contained in a population of non-adherent, Fc-receptor bearing lymphocytes which also contained the ADCC effector cell. Thus in both experimental aniamls and man the SCMC effector was shown to possess Fc-IgG receptors. Studies were also undertaken to assess the cytotoxic potential of T-cells and various T-cell sub-populations. In previous studies employing human peripheral blood lymphocytes and allogeneic Chang cell targets, MICC had been shown to be T-cell mediated. Additional studies were undertaken to determine if MICC was an exclusive property of either Tμ or Tγ subset of T-cells. As defined by surface receptors for sheep erythrocytes (SRBC), MICC was mediated solely by T-cells possessing such receptors and was not mediated by cells (B-cells, K-cells) lacking SRBC receptors. Moreover MICC was mediated by T-cells possessing Fc receptors for IgG (Tγ) and those possessing Fc receptors for IgM (Tμ). Thus T-cell mediated MICC was not an exclusive property of either human peripheral blood T-cell subset. Having thus demonstrated that Both Tμ and Tγ sub-populations were capable of cytolytic activity in an MICC assay employing non-immunized effector cells, studies were undertaken to investigate the cytotoxic activity of Tμ and Tγ cells in cell mediated lympholysis (CML). In such assays, immune cytolytic lymphocytes are generated in vitro during one-way mixed leukocyte reactions. In such cultures, cytolytic precursors are differentiated into mature CML effector cells. These immune CML effectors possess cytolytic specificity for target cells sharing HLA-A, B or C locus antigens with the stimulator cells employed to induce the mixed leukocyte reaction. At the precursor level, T-cells were shown to contain the CML precursor and among T-cell subsets, those populations effectively enriched for Tμ cells generated CML effectors while those populations enriched for Tγ cells did not. At the CML effector level, cytotoxic effectors were again shown to be T-cells and the CML effector was contained in a T-cell subset which did not express Fc-IgG or Fc-IgM receptors and Tμ and Tγ cells did not mediate CML. Thus while both Tμ and Tγ cells possessed non-immune cytolytic effector potential as evidenced by their ability to mediate MICC, Tμ cells but not Tγ cells contained the cytolytic precursor for immune CML and once differentiated into a mature CML effector these cells did not express Fc-IgG or

Fc-IgM receptors. Further studies were undertaken to examine the cytotoxic phenomenon of "hapten-modified self" in humans. In experimental animals such assays involving sensitization to chemically (TNP) or virus (influenza) "modified" autologous cells result in cytotoxic effectors which are hapten specific and genetically restricted to lyse only those targets sharing serologically defined major histocompatibility complex (MHC) antigens with modified stimulator cell and/or the responding cytolytic effector. Two such assays have been established employing human peripheral blood lymphocytes and either chemically (TNP) or virus (influenza) modified stimulator and targets. T-cells have been shown to mediate cytotoxicity in humans in both assays. Studies of the specificity of TNP-dependent CML (TDCML) have thus far shown that in contrast to experimentzl animals, lysis of TNP-modified targets is not restircted to those targets sharing serologically defined MHC gene products with the modified stimulator and responder. Although a portion of the lytic effector activity is directed at TNP in association with polymorphic HLA-A, B, and C locus antigens, additional cytolytic specificities are directed toward TNP in association with non-polymorphic "public" specifications present in most individuals. Similar specificity studies are underway in the influenza modified-self assay.

Investigation of the Role of Immunologic Responses in Gastrointestinal Diseases - Two hepatic diseases, chronic active hepatitis (CAH) and primary biliary cirrhosis (PBC) are associated with a variety of immunologic phenomena including hepatic necrosis and infiltration of the liver with chronic inflammatory cells including lymphocytes. To test the hypothesis that liver damage in these two conditions might result from cytotoxic reaction directed at liver-specific antigens, peripheral blood lymphocytes from CAH and PBC patients were tested for SCMC, MICC and ADCC against Chang liver cell targets in vitro. SCMC was observed in both patient groups and controls but cytotoxicity was not specific for Chang liver cells. SCMC in CAH, PBC and controls was not T-cell mediated but K-cell mediated. In patients with PBC but not CAH, SCMC but not ADCC or MICC for Chang targets was reduced compared to controls. Thus while the effector cell(s) mediating SCMC and ADCC were not separable on the basis of surface characteristics, dissociation of these effector activities was observed in a disease state, PBC. The observation that patients with CAH and PBC were not found to have increased killing of Chang liver cells in vitro suggests that at least as assessed with this cytotoxicity assay, hepatic-specific cytotoxic lymphocytes are not present in the peripheral blood of such patients.

The investigation of a patient with IgA deficiency, villous atrophy, and IgG anti-epithelial cell antibodies in his serum suggested that K-cell mediated ADCC directed against gastrointestinal epithelial antigens might play a role in the pathogenesis of villous atrophy in a variety of gastrointestinal diseases. Studies were therefore undertaken to define the cytotoxic repertoir of small intestinal mucosal lymphocytes in experimental animals. Techniques were developed to obtain two populations of lymphoid cells from the small intestine of guinea pigs: the first consisting of lymphocytes from within the epithelium, termed intraepithelial lymphocytes (IEL); and the second from the lamina propria, termed (LP). Both IEL and LP were found to possess cytotoxic effector function, the former mediating SCMC, MICC, and ADCC and the latter mediating MICC but not SCMC or ADCC. Thus in experimental animals, effector lymphocytes capable of mediating cytotoxicity were demonstrated in the mucosa of the small intestine. Such effector cells may be important in the normal local immune response of the small intestine as well as in the pathogenesis of certain inflammatory diseases involving the small bowel.

Proposed Course: Studies using well characterized assays to evaluate cytotoxic effector function of lymphocytes from patients with immunodeficiency diseases, autoimmunity and neoplasia will be continued. Particular emphasis will be placed on the newly defined assays of TDCML and influenza-dependent CML. Studies will be directed toward defining the T-cell recpetor for antigen on such cytolytic T-cells and determining if immune response genes modulate these cytolytic responses. Ongoing studies to determine the origin and functional capabilities of K-cells will be continued. Further studies on the immunologic reactivity of IEL and LP cells from the small intestine of guinea pigs will be undertaken including studies of immunoglobulin isotype distribution and secretion, regulation of immunoglobulin biosynthesis and studies of the precurosrs of immunoglobulon secreting cells within the lamina propria.

Publications:

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Shaw, S., Nelson, D.L., and Shearer, G.M.: Human cytotoxic response *in vitro* to trinitrophenyl-modified antologens cells. I. T-cell recognition of TNP in association with widely shared antigens. *J. Immunol.* In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
NCI-Z01-CB-04018-09-MET

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Study of Human Immune Defense Mechanisms and Its Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	E. Clinton Lawrence, M.D.	Clinical Associate	MET	NCI
	Samuel Broder, M.D.	Investigator	MET	NCI
	R. Michael Blaese, M.D.	Senior Investigator	MET	NCI

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LAB/BRANCH
Metabolism Branch

SECTION
Cellular Immunology Section

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are aimed at understanding how the human immune system recognizes and destroys foreign cells. Special emphasis is placed on early antigen non-specific cytotoxic responses, which precede specific immune recognition. Also, we have studied membrane bound Fc receptors with specificity for a given immunoglobulin. Each model offers the potential to disect apart critical mechanisms involved in immune defense.

Project Description:

Objectives: The objectives of these studies were to (1) delineate the importance in man of a newly recognized system of non-specific cell mediated spontaneous cytotoxicity, (2) to characterize a newly recognized class of surface receptors for the Fc portion of human immunoglobulin on human T cells, and (3) to examine the phylogeny of antigen non-specific cytotoxicity systems in vitro.

Methods Employed: In vitro cellular cytotoxicity systems have been developed for antibody dependent, mitogen induced, cell mediated lympholysis, and spontaneous cytotoxicity using a sensitivity micro ^{51}Cr release assay for various target cells. These results were correlated with standard in vitro and in vivo assays of cell mediated and humoral mediated immunity. For the characterization of immunoglobulin class specific Fc receptors on human T cells purified IgG, IgM and IgA were obtained by combinations of ion-exchange chromatography, affinity chromatography, gel filtration and antigen specific absorption of certain monoclonal mouse myeloma proteins.

For the phylogenetic studies an articicial sea water mixture was used as holding medium for viable organisms prior to use.

Major Findings: It is widely recognized that the immune system of vertebrates is capable of responding to foreign challenge with exquisite specifity. Furthermore, a primary antigenic challenge usually leads to the generation of longed lived specific immunologic memory cells. Such specifity however, required complex cellular interactions and amplification steps which requires from days to weeks to become fully operative. These long time constraints mean that early host defense must occur prior to the generation of classical specific responses and is probably quite non-specific. Our laboratory has had a major interest in evaluating in vitro assays of rapidly acting non-specific cell mediated cytotoxicity. We believe these models represent important measures of early non-specific host defense.

Two unique models of non-specific cytotoxicity have been developed. One model measures pure monocyte mediated killing. Development of cytotoxicity is due to an in vitro inactivation of a potent suppressor cell found in fresh cells. Interestingly several widely used stimulatory plant mitogens (especially PWM) act to dramatically stimulate cellular suppression of non-specific monocyte mediated cytotoxicity. Our expanding interest in this unique model stems from several observations. First, the model allows a potentially fruitful approach to study the negative regulatory influences which control cytotoxic monocyte function in vitro and in vivo. Second, this type of antigen non-specific non-immune monocyte killing is quite analogous to natural killing systems involving K cells and evidence is accumulating from numerous labs that this type of early non-specific killing may be a very important first line of defense against both infections and malignant diseases. Third, we have discovered that our spontaneous killing model varies immensely with various disease states. For example, patients with acute viremia and certain patients with lymphomas are super killers and kill without the necessity of pre-culturing. Our data suggests that these patients lack circulating suppressors. Other patients, however, lack the ability to kill. Patients with CLL cannot kill due to a lack of circulating monocytes. The fact that patients with the Wiskott-Aldrich syndrome cannot kill is an independent confirmation of their previously described monocyte killing defect. Certain immunodeficient patients including a subset of common variable hypogammaglobulinemic patients and all patients with ataxia-telangiectasia fail to kill.

At least some of the hypogammaglobulinemic patients fail to kill as a result of hyperactive suppressor cell functions. A second model of nonspecific early host defense is dependent upon the presence of a non-antibody heat labile substance found in normal human plasma which can arm T cells as well as monocytes to kill. The nature of this plasma factor is under active investigation and shares many properties with early factors of the alternate complement pathway. This finding can potentially unify non-cell mediated and cell mediated non-specific antibody independent *in vitro* models of cytotoxicity since it implies a common mode of activation.

Our lab has also had an interest in the phylogeny of non-specific responses since intertebrates largely lack specific immunologic recognition capabilities. In fact, we have recently shown that invertebrate mononuclear cells can kill a variety of malignant and normal mamallian cell lines.

A final area of interest in our lab concerns the ability of various human mononuclear cells to bind immunoglobulin through an Fc receptor. We have developed evidence that human T cells posses a receptor for IgA as well as previously described receptors for IgG and IgM. This new data suggests that T cells distinguish different classes of immunoglobulin by differences in their Fc receptor. Such data is entirely consistent with our previously described model in the chicken of class specific suppressor T cells capable of modulating class specific immunoglobulin production (IgG, IgA, IgM) by B cells.

Significance to Biomedical Research: These present studies extend our understanding of the way in which a host organism is able to cope with foreign grafts whether they are bacterial, malignant, or viral in origin. Our studies of non-specific spontaneous cytotoxicity suggest that it may play a central role in host defense and act long before specific mechanisms of immunity can be mobilized.

Data from selected patient groups suggest that these models are sensitive gauges of *in vitro* and *in vivo* immunocompetence. Our data concerning class specific Fc receptors found on T cells allows us to dissect out what role various subclasses of human T cells play in the generation of a specific immune response. Furthermore, we have begun to correlate the presence of absence of these various receptors in a large group of immunodeficient patients.

Publications:

Muchmore, A.V., Decker, J.M. and Blaese, R.M.: Spontaneous cytotoxicity of human peripheral blood mononuclear cells towards RBC targets. I. Characterization of the killer cell. *J. Immunol.* *119*:1680, 1977.

Muchmore, A.V., Decker, J.M. and Blaese, R.M.: Spontaneous cytotoxicity of human peripheral blood mononuclear cells towards RBC targets. II. Time dependent loss of suppressor cell activity. *J. Immunol.* *119*:1686, 1977.

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Blaese, R.M., Lawrence, E.C., Muchmore, A.V., Grayson, J., Dooley, N.J. and Koski, I.: Immunoglobulin secreting cells in the peripheral blood and in mitogen stimulated cultures from patients with immunodeficiency diseases: *In Vitro Induction and Measurement of Antibody Synthesis in Man.* Edited by A.S. Fauci and R.E. Bollieux, Academic Press, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB- 04019-09-MET

PERIOD COVERED
October 30, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunoregulatory Cells in Human Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Metabolism Branch

SECTION

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TOTAL MANYEARS: 5	PROFESSIONAL: 4	OTHER: 1

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to investigate the immunoregulatory properties of malignant lymphocytes. We are currently determining the helper and suppressor activity of neoplastic T-cells from patients with acute lymphoblastic leukemia and the Sézary syndrome. In addition, we are attempting to characterize normal and neoplastic T cells on the basis of their receptors for the Fc piece of IgM and IgG.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: A number of essential insights into the nature of the humoral immune responses resulted from the study of neoplasms of the B cell/plasma cell series. The recognition that so-called paraproteins derived from patients and certain animals with multiple myeloma (a malignant proliferation of plasma cells) represented extremely homogenous immunoglobulins has affected essentially every phase of immunologic research. An understanding of antibody structure, function, metabolism and genetics depends heavily on experiments based on the use of such homogenous immunoglobulins. Indeed, certain immunoglobulin classes (such as IgD and IgE) would have been difficult or impossible to evaluate without the availability of appropriate paraprotein classes. Recently recognized membrane and functional differences between B cells and T cells provided an important basis for classifying neoplastic lymphocytes. Such a classification has already proved to have therapeutic and prognostic relevance in characterizing lymphomas and certain leukemias. The study of malignant T cells and their products may prove to be as important in answering questions regarding cellular immunity, and especially, the T cell regulation of humoral immune responses, as myeloma cells and their paraprotein products have proved to be in answering questions concerning the immunoglobulin synthesizing system. During the past year, we have focused a considerable degree of research and clinical effort in studying patients with acute and chronic leukemias thought to be of T-cell origin.

T cells play a critical role in a number of immune systems. These cells are involved in cell-mediated immunity which includes such phenomena as delayed hypersensitivity, allograft rejections, graft vs. host reaction, tumor cell killing in some systems, and lysis of virally-infected target cells. Normal T cells proliferate in vitro in the presence of a number of plant mitogens such as phytohamagglutinin and concanavalin A. They may mediate the mixed-lymphocyte-reaction, act as killer cells in certain models of lymphocyte-induced cytotoxicity, and generatecertain soluble effector proteins such as lymphokines. In addition, T cells play an indespensible role in regulating humoral immune response by acting as potentiators (helper cells) or inhibitors (suppressor cells) of the transition of B cells into immunoglobulin producing plasma cells. Both antigen-specific and nonspecific helper and suppressor functions have been identified.

During the past year we have extended our analysis of the immunoregulatory properties of leukemic T cells from patients with Sézary syndrome*, and

*The Sézary syndrome is a T-cell leukemia in adults characterized by severe erythroderma, diffuse lymphadenopathy, and circulating neoplastic cells with cerebriform nuclei.

we have given special attention to the leukemic T cells of some patients with childhood lymphoblastic leukemia. We have previously shown that some (but not all) patients with the Sézary syndrome may have leukemic cells which act as so-called helper T cells. By refining our analysis we have learned that approximately 5 of 12 patients studied had helper activity in a system where any contaminating residual normal T-cell function was mimimized by dilution studies. We have also discovered a patient with acute lymphoblastic leukemia whose neoplastic cells acted as T cells that could perform a helper function. Furthermore, we found another patient with acute lymphoblastic leukemia (also T-cell type) who had hypogammaglobulinemia prior to chemotherapy and whose neoplastic cells appeared to function as pro-suppressor cells. Such cells required a T-T interaction for maximal suppressor effect.

Methods Employed: To study the transition of circulating lymphocytes into immunoglobulin secreting plasma cells, peripheral lymphocytes were cultured in the presence of pokeweed mitogen or in some cases a water soluble mitogen derived from a Nocardia opaca. The amount of IgM, IgA and IgG synthesized and secreted into the media was determined by double-antibody radioimmunoassays. The presence of circulating suppressor cells was assayed by a co-culture technique. Circulating neoplastic T cells from various patients and peripheral blood lymphocytes from normal control individuals were co-cultured. The synthesis of immunoglobulin by cells of the two subjects in this co-culture was related to the sum of the expected contribution of each population. In order to assay for helper activity, we took advantage of the observation that highly purified B cell populations, freed of T cells, synthesize very small quantities of immunoglobulin in the presence of pokeweed mitogen. (It should be noted that pokeweed mitogen is a highly T cell dependent activator, whereas Nocardia opaca is a relatively thymic independent polyclonal activator, and this will be discussed further below.)

Major Findings: The neoplastic lymphocytes of essentially all patients with Sézary type T-cell leukemias failed to produce immunoglobulin under any of the conditions assayed. None of the Sézary patients studied had neoplastic T cells which exhibited suppressor cell activity. Indeed, on a number of occasions, normal lymphocytes showed a greatly amplified immunoglobulin synthesizing and secreting capacity in the presence of Sézary cells compared to their capacity when cultured alone. The neoplastic cells from 5 of the 12 patients with the Sézary syndrome studied functioned as helper T cells in a system where dilutions were made to exclude a role for residual normal T cells. Thus, there was a 6 to over 30-fold augmentation of immunoglobulin production by normal purified B cells after the addition of Sezary cells. We observed a comparable helper activity in the neoplastic T cells from a child with acute lymphoblastic leukemia. These studies indicated that under some conditions neoplastic T cells may act as though they originate from T cells committed to mediate helper activity.

Our analysis of another patient with acute lymphoblastic leukemia and hypogammaglobulinemia prior to chemotherapy may clarify the role of T-T interactions in the regulation of normal humoral immune responses. The neoplastic T cells from this patient acted as potent suppressors of pokeweed mitogen induced immunoglobulin production by co-cultured normal unseparated lymphocytes. However, in experiments in which purified normal B cells, purified normal T cells, and purified irradiated normal T cells were used in various combinations, we observed that this child's neoplastic T cells required a radiosensitive subset of normal T cells to exert their suppressor activity in the presence of pokeweed mitogen. Furthermore, we found that normal T cells when stimulated with pokeweed mitogen released soluble factor(s) within 24 hours of culture, which activated these leukemic cells to suppress immunoglobulin production without the additional requirement of cooperating normal T cells. In further studies, we learned that the presence of cooperating normal T cells and pokeweed mitogen together was essential in the suppression of Nocardia water soluble mitogen-driven immunoglobulin production. It should be re-emphasized that pokeweed mitogen is a relatively thymic-dependent polyclonal activator, whereas Nocardia water soluble mitogen is a relatively thymic-independent polyclonal activator. Taken together, these results suggest that the leukemic cells under discussion acted as prosuppressor cells requiring the cooperation of a normal amplifying subset of T cells in order to emerge as suppressor effectors. To our knowledge this represents the first demonstration of an interaction between normal and neoplastic cells in the generation of immunoregulatory effector cells.

In collaboration with Dr. Dean Mann, an analysis of the membrane markers expressed by these pro-suppressor leukemic cells was undertaken. We observed that approximately 30-40% of the circulating neoplastic T cells on this patient expressed Ia-like membrane determinants. The Ia-like determinants were detected by B-cell alloantisera as well as heteroantisera. As these leukemic cells became activated from the pro-suppressor to the suppressor effector stage, there was a loss of these Ia-like membrane determinants.

Our studies of this pro-suppressor like leukemia permitted us to construct a model for the induction of human suppressor effector cells. In this model, percursor T cells (pro-suppressor cells) must interact with a different activating set of T cells before maturing into the suppressor effector cells. The model developed from the use of these cells provides at least two testable expectations for future research. The first is that neoplastic diseases represent proliferation at different steps in the suppressor cell differentiation network. Second, patients with neoplasms composed largely of suppressor effectors will have hypogammaglobulinemia directly related to tumor cell burden; however, those with neoplasms of pro-suppressors or suppressor activators will have hypogammaglobulinemia related to the residual number or functional capacity of non-neoplastic cooperating T cells as well as the leukemic cells.

Another area of interest during the past year has been the characterization of receptors for the Fc piece of IgG and the Fc piece of IgM on human T cells and B cells. These studies were done by Dr. Werner Pichler. Fc receptors were detected by using appropriate immunoglobulins with antibody activity against ox red cells. The enumeration of rosette forming cells under the proper conditions could be used as an index for cells which express receptors for the relevant Fc piece. There has been a widely held belief that those T cells which act as positive regulators of humoral immune responses (helper cells) express receptors for the Fc piece for IgM. Such cells are conventionallyreferred to as Tμ. A corollary of this widely held belief is that those cells which act as negative regulators of humoral immune responses (so-called suppressor cells) express receptors for the Fc portion of IgG. Such cells are conventionally referred to as Tγ. The concept of two distinct populations (Tμ and Tγ) with different immunoregulatory potential was of such clinical and theoretical importance that we have devoted a substantial portion of research time and effort in pursuing this issue. Our results to date have shown that rigid classification of T cells into Tμ and Tγ cells is not appropriate. We have observed that normal T cells may, under certain circumstances, express both Fc IgM and Fc IgG receptors simultaneously. Furthermore, there is a transition of so-called Tγ cells into Tμ under certain conditions. The signal for this transition appears to be an interaction with human complexes. In a further series of studies, it became evident that it is not possible to simply assign an immunoregulatory function to T cells on the basis of the Fc receptor expressed. Under some conditions Tγ cells may function as helper cells and Tμ cells may function to inhibit certain immune reactions.

We have also attempted to characterize the presence of Fc-IgM and Fc-IgG receptors on normal B cells, neoplastic B cells, and long term tissue culture lines of human B cells. We have observed that these cells may express both Fc IgM and Fc IgG receptors. Furthermore, the secretion of immunoglogulin is not a function of the kind of Fc receptor expressed B cell. We are currently trying to evaluate the role of Fc IgG and Fc IgM receptors in various forms of mitogen-induced, antibody dependent, and spontaneous cell-mediated target killing system.

Significance to Biomedical Research: The recognition that some T-cell leukemias (or lymphomas) may represent a homogenous proliferation of T cells which are programmed for either helper or suppressor function is important for a number of reasons. First, certain forms of humoral immune deficiency may be due to a helper cell deficit, and not totally due to an intrinsic defect in B cells alone or excessive numbers of suppressor cells. It is possible that certain classes of neoplastic cells or soluble products produced by such cells could prove useful in the treatment of immune deficient states due to thymic dysfunction. Similarly, if such helper cell factors could be isolated (or synthesized), they might be useful in those situations where it is desirable to amplify a normal immune response, for example, following immunizations or in overwhelming infections.

In mice, it has been possible to show that there exists a genetically controlled Ly-system of membrane markers which may corelate with helper or suppressor function. Three groups of T cells have been identified in mice. One population bears all three of the so-called Ly determinants (Ly-1^+,2^+, 3^+). Another population bears only Ly-1 determinants and a third population bears Ly-2,3 determinants. It has been demonstrated that those cells which are Ly 1^+ act as helper cells under some conditions. In contrast, those cells which are Ly 2, 3^+ act as suppressor cells under some conditions. In addition, in mice there is a subregion of the I portion of the major histocompatability complex (designated IJ) which may in code for the suppressor cell function and certain suppressor cell factors. These IJ encoded factors may inhibit both humoral and cellular immune responses in a number of systems. Furthermore, antisera raised against IJ in coded products may potentiate certain immune responses. Such anti-IJ antisera may apparently promote tumor rejection in certain systems presumably by eliminating suppressor cells which interfere with host immune responses directed against lethal tumors. The use of neoplastic T cells as starting reagents for the development of appropriate antisera or the isolation of various factors may make it possible to establish a serological marker system for human helper and suppressor function, and may make it possible to serologically manipulate immune responses in certain cancer patients to potentiate tumor rejection. The use of such neoplastic T cells may also provide a convenient probe to analyze human B cell subsets and to elucidate more fully human T-T interactions.

Proposed Course: During the coming year a continuing effort will be made to characterize the immunoregulatory properties of lymphocytes from patients with T cell leukemias and lymphomas. A very high priority will be given to the development of antisera or possible pharmacologic agents which have selective activity for human helper or suppressor T cell subsets. In addition, we will continue to characterize the Fc IgM and Fc IgG receptors found on human B cells and T cells in the hope of defining the biological role for these membrane determinants. We are also extending these studies to an evaluation of Fc IgM and IgG receptors in so-called K cells (cells which mediate antibody dependent cellular cytotoxicity). We will investigate whether it is possible to isolate soluble factors from malignant lymphocytes which can mediate the immunoregulatory activities of intact cells.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER NCI-Z01-CB-04020-09-MET

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Natural Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jay A. Berzofsky, M.D., Ph.D.	Expert	MET	NCI
OTHER:	David H. Sachs, M.D.	Senior Investigator	I	NCI
	David Pisetsky, M.D.,Ph.D.	Clinical Associate	I	NCI

COOPERATING UNITS (if any)
Immunology Branch, NCI

LAB/BRANCH
Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.0	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antisera raised in different inbred strains of mice to two natural protein antigens under H-2 linked Ir gene control, sperm whale myoglobin and staphylococcal nuclease, have been studied for their specificity for different determinants on the antigen molecule. In the case of nuclease, H-2-linked Ir genes affect the proportion of antibodies to different determinants. The H-2 linkage of this phenomenon has been confirmed in an F_2 study, and testing of additional strains suggests the existence of multiple genes in H-2 which have yet to be mapped. In the case of myoglobin, two Ir genes have been mapped to the I-A and I-C subregions of H-2. Recombinant strains with only the gene in I-C ($Ir\text{-}M^b\text{-}2$) produce an intermediate level of antibodies to whole myoglobin, lacking antibodies to the C-terminal region (132-153), whereas strains with only the gene Ir-Mb-1 in I-A make antibodies to this region. These results suggest that the responses to different determinants on the same antigen molecule are controlled independently by different genes, and that intermediate responsiveness is only the net effect of high responder genes for some determinants and low responder genes for other determinants.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The primary objective has been to defind the mechanism by which Ir (immune response) genes control the antibody response to natural protein antigens, as opposed to the synthetic polymers and hapten-carrier complexes more widely studied, with the hope of understanding the modes of recognition involved at this regulatory level, the cells which participate, and the cell membrane receptors which mediate the regulation.

Methods Employed: Fragments of sperm whale myoglobin were prepared by CNBr cleavage and chromatographic purification. Synthetic peptides corresponding to supposed antigenic determinants of myoglobin were prepared by the Corley-Sachs-Anfinsen rapid modification of the Merrifield solid phase peptide synthesis method, and were extensively purified. TNP-Myoglobin was prepared using trinitrobenzenesulfonate. Staphylococcal nuclease was attached to larger "carrier" proteins by use of a bifunctional coupling reagent, m- maleimidobenzoyl N-hydroxysuccinimide ester, one side of which reacts with amino groups and the other with sulhydryl groups. Myoglobin and its fragments were labeled specifically at the N-terminal alpha-amino group either by carbomoylation with $K^{14}CNO$ or by acylation with N-succinimidyl-(2,3-^{3}H) propionate. Antibody concentrations specific for these fragments were measured from the plateaux of saturation binding curves as a function of increasing antigen concentration using polyethylene glycol to separate antibody-bound antigen from free antigen, as previously described. Mouse spleen cells were separated into glass-adherent and nonadherent populations, using EDTA (1 mM) at 37°C to remove adherent cells from the glass.

Major Findings: The H-2-linkage of the Ir gene control of the antibody response to sperm whale myoglobin was confirmed by a formal analysis of the responses of the progeny of a backcross of high (B10.D2) x low (B10) responder F_1 mice to the low responder parent, and H-2 typing of the mice by cytotoxicity. In conjunction with this, high responsiveness was found to be dominant in the F_1 hybrid. The existence and mapping of two distinct Ir genes within the I region of the mouse H-2 major histocompatibility complex, one in the I-A subregion and the other probably in the I-C subregion, was extended with the statistical study of different mice of congenic resistent strains, including a newly developed strain, the B10.GD, a gift of Dr. M.E. Dorf. The existence of two or more genetically separable Ir genes in H-2 for the same antigen, with some recombinant strains having only one of the genes being intermediate responders, allowed us to ask the question whether the intermediate responders are such because they have genes which allow them to respond to some of the determinants but lack genes necessary for response to other determinants; that is, can different determinants on the same antigen molecule be under the independent control of different H-2 linked Ir genes? Such a result would explain our earlier results with staphylococcal nuclease, described previously, and would contradict the predictions based on experiments with hapten-carrier model antigens. The hypothesis was tested by measurement of the amount of anti-myoglobin antibody made by these intermediate recombinant strains, and their high and low responder parental strains, specific for different fragments of myoglobin. It was found that both intermediate strains with only Ir-Mb-2 (the gene I-C) are indistinguishable from their low responder parental strains in the amount of antibody binding to fragment (132-153), even though they make 4 to 6-fold more antibody to the molecule as a whole. In contrast, the strain which has Ir-Mb-1 (the gene in I-A), makes just as much antibody specific for the region (132-153) as its congenic high responder parental strain with both genes. Thus, the prediction of the hypothesis that the response to different determinants are under the control of different genes is so far borne out. To prove the hypothesis, we need to find a region to which the intermediate responders behave as high rather than low responders. The insolubility (under the conditions of assay) of the two other CNBr fragments of myoglobin has hindered our efforts. In addition to working on other assay methods, we have synthesized by the solid phase method one 8-residue and one 10-residue peptide corresponding to two different determinants on myoglobin defined by Atassi as reacting with goat antisera to myoglobin. Despite extensive purification and characterization of these peptides, we have been unable to confirm Dr. Atassi's findings that these regions react with goat or sheep antisera, let alone mouse antisera, by several different assay methods including the inhibition of quantitative precipitin method used by Atassi. Thus, despite our inability to use these peptides to study Ir gene control, we have thrown into question some of the earlier delineation of myoglobin antigenic determinants by Atassi. In addition, several preliminary experiments have been performed to measure the binding of highly radioactive myoglobin to fractionated spleen cell populations of high and low responder strains. However, the problem of high background levels of "nonspecific" binding has so far hindered progress. Finally, antibodies to TNP on the immunogen TNP-myoglobin have been found to be under H-2-linked Ir gene control, even though antibodies to native myoglobin are not made.

Three other studies were carried out as a continuation of the work on staphylococcal nuclease as a model antigen. First, in order to be sure that the differences previously described between the supposedly congenic strains B10 and B10.A were due to H-2-linked differences rather than genetic drift in the supposedly identical immunoglobulin structural genes, and F_2 generation of A/J x B10 was bred, and animals selected which bore the $H\text{-}2^a$ haplotype of the A/J parent but were homozygous for the $Ig\text{-}1^b$ heavy chain allotype of the B10 parent. These animals were found to be capable of responding to nuclease with antibodies to the region (99-149) typical of the B10.A strain, rather than the B10, indicating that genetic drift in immunoglobulin genes could not be the explanation of our observations.

Second, an attempt was made to see whether the genetic control of the response to individual determinants of nuclease could be overcome by immunizing with nuclease attached to a larger immunogenic carrier. When nuclease failed to bind adequately to acetylated bovine serum albumin (BSA), a promising heterobifunctional reagent was found (see Methods) to link the alpha-NH_2 of nuclease specifically to SH groups attached to BSA or keyhole limpet hemocyanin (KLH) with another bifunctional reagent, methyl-4-mercaptobutyrimidate. However, the specificity of the maleimido group of the first reagent for SH groups was insufficient to prevent cross-linking and precipitation of the nuclease itself. The covalent complexes formed with BSA or KLH were large and poorly soluble and no adequate stoichiometry could be determined. Thus, other methods will be required before this study can be carried out.

Finally, in view of the evidence we had obtained with Ron Schwartz, Alan Schechter, and David Sachs that the T-cell proliferative response to nuclease was probably under the control of multiple H-2 linked genes, a reassessment was begun, in collaboration with David Pisetsky and David Sachs of the original mapping of the Ir-Nase gene controlling the antibody response to a single genetic site in the I-B subregion. In addition to measuring the total antinuclease levels, the ratio of antibodies specific for the regions (99-149) and (1-126) was also determined for each strain. Recombinant strains B10.A (5R) and B10.A (4R), which were originally considered "low responders," were found to have a ratio of antibody response more like the B10.A high responder than like the B10 low responder. Moreover, another high responder, B10.D2, showed a ratio different from the B10.A type originally defined. Thus, the genetic controls on the antibody response to nuclease are much more complicated than the original one-gene hypothesis supposed. Further mapping studies are in progress.

Lastly, it was demonstrated mathematically that the affinity of antibodies could be determined directly from the midpoint of a radioimmunoassay plot of bound/free vs. free antigen, whereas if bound/free were plotted vs. total antigen as is commonly done, the midpoint would differ from $1/K_A$ by half the antibody binding site concentration, a significant error term.

Significance to Biomedical Research: The control of different determinants on the response to same antigen molecule by genetically distinct Ir genes places important constraints on the mechanism by which Ir genes regulate the immune response. This result is inconsistent with the the hypothesis that helper T cells mediate Ir gene control through recognition of a carrier-like portion of the molecule and suggests that natural protein antigens cannot be forced into the hapten-carrier mold. Moreover, it is more easily explained by Ir gene function in effector cells such as B cells or proliferating T cells. It suggests that Ir gene control of very complex multideterminant antigens may not have been detected because failure to respond to one determinant would not be missed. It is hoped that understanding of the genetic regulation of the immune response will ultimately lead to an understanding of diseases in which this response is deficient or hyperactive to specific antigens, as in malignancy or autoimmune diseases, respectively. It may also lead to methods to manipulate the immune response selectively where necessary, as in organ transplantation.

Proposed Course: Further studies of the specificity of genetically controlled response to myoglobins and nuclease are underway to confirm and extend the conclusions discussed above. In particular for myoglobin, other synthetic and natural fragments are under study using new assay procedures, such as a solid phase assay, to overcome problems mentioned. In addition, myoglobins of other closely related species that differ in only a few amino acids are under study. Monoclonal antibodies to myoglobin are to be raised in hybridoma cell lines to prepare antiidiotype reagents. Also, the myoglobin-response system is now sufficiently developed that approaches are underway to look at the control of the response *in vitro* in order to study the subpopulations of lymphoid cells involved. Finally, studies are planned to isolate antigen-specific receptors from T-cells to compare their specificity with that of *Ir* gene control.

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SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
October 1, 1977 to September 30, 1978

The diagnostic and research activities of the staff of the Laboratory of Pathology are listed by Sections. The research accomplishments are especially impressive when one considers that the majority of the staff participate in the diagnostic services for Clinical Center patients. These services include surgical pathology, postmortem pathology, diagnostic cytopathology, electron microscopy and the isolation of certain infectious agents from human tissues. Applications of newer methods such as flow microfluorometry and cell sorting to the diagnosis and study of human disease have also increased. Dr. José Costa, Acting Head of the Surgical Pathology and Postmortem Section, and Dr. Bruce L. Webber supervise the residents and the staff pathologists who participate in the diagnostic services. They have made changes in conferences and in surgical pathology and postmortem reports to improve the residency training program and the diagnostic services for Clinical Center patients. It is by offering opportunities and facilities for laboratory research that we have been able to recruit and retain an outstanding group of anatomical pathologists who have given vital support to the clinical programs of NCI as well as all the other Institutes with patients in the Clinical Center.

In April, 1978 the Image Processing Unit was transferred from the Quantitative Cytology Section to the Office of the Chief. Earlier in the fiscal year, Dr. Donald E. Henson was assigned to the Office of International Affairs of the NCI. Dr. Raul Braylan resigned from the Hematopathology Section on July 31, 1977 to take a position as Hematopathologist at the University of Florida Medical School, Gainesville, Florida.

The research programs of the Laboratory have involved:

A. Hematopathology and Immunopathology
B. Electron Microscopy and Histochemistry
C. Viral Oncology and Cell Biology
D. Comparative Pathology and Carcinogenesis
E. Development and Use of Automated Equipment for Cytology Diagnoses
F. Clinico-Pathologic Studies on a Variety of Human Diseases

Some of the significant research and clinico-pathologic accomplishments in which the staff of the Laboratory participated, either as principal investigators or as collaborators include:

(1) Further characterization of the structure and biochemistry of the nucleic acid of the murine K virus and evaluation of its relationship to other known papovaviruses.

(2) Demonstration that the same oncogenic virus (polyoma) acting on the same target cells (mouse salivary gland epithelium) can induce morphologically and biologically different neoplasms if microenvironmental conditions

(*in vivo* or *in vitro* transformation with or without stromal tissue) are varied during and/or after the transformational events. The data suggest that the primary transformational events are the same, but that microenvironmental factors are important in modifying tumor progression, differentiation, growth and degree of anaplasia and ability to invade and metastasize.

(3) Further investigation, using animal models, of pulmonary carcinogenesis caused by air pollutants, tobacco smoke condensates and particulate matter; e.g. asbestos and fibrous glass.

(4) Further characterization of the morphology, immunoglobulin production and surface membrane characteristics of subtypes of human lymphomas and leukemia. Certain correlations between these findings and the biological behavior of the neoplasms and response to therapy have been established.

(5) Identification of two populations of lysosomes in normal human fibroblasts and elucidation of mechanisms of secretion and resorption of lysosomal enzymes. This work will lead to a better understanding of the pathogenesis of certain genetic diseases as well as rheumatoid arthritis.

(6) Development of a radioimmunoassay system to identify oligosaccharides in urine. The amount of Le^b active oligosaccharides was increased during pregnancy and lactation and was correlated with different Lewis blood types. This and other experiments, using fluorescence labelling of glycoconjugates, will lead to a better understanding of the function and fate of cell surface glycoproteins.

(7) Development of techniques for quantitative analysis of flow microfluorometric data which can be used in automated screening of human cervical-vaginal cytologic material. Another development in FMF experiments is to use chicken red cells as an internal biological standard for assessing changes in DNA content or its structural alteration produced by chemotherapeutic agents. These techniques hold considerable promise for automated cytologic diagnosis and for assessing the effects of therapy on cellular kinetics of specific types of leukemia and lymphoma.

(8) Further studies of the structure, topology and function of Fc receptors induced by herpesviruses.

(9) Studies of the relevance of the polypeptide marker to neurovirulence of type 1 and type 2 poliovirus vaccine were continued. Viruses isolated from the gut of a child fed type 1 poliovaccine were demonstrably more neurovirulent for monkeys than reference vaccine regardless of whether the isolated virus strains had undergone mutations in the largest virion polypeptide, VP1. For type 2 poliovirus vaccine (virus isolated from gut and central nervous system of a case of vaccine-associated paralysis in an immunodeficient child) the preliminary results were consistent with the hypothesis that vaccine mutations rather than host factors alone may have

been responsible for the paralysis.

Many members of the Staff, in addition to their laboratory and service work, serve on numerous national and international committees, editorial boards, etc. Some of the principal activities of this sort are: Dr. C.J. Dawe, Project Officer for the Registry of Tumors in Lower Animals (Smithsonian Institution), and Associate Editor of Cancer Research; Dr. C.J. Herman was Co-chairman of DCBD Committee on Cytology Automation until the contract program was reorganized; Dr. L.B. Thomas on ad hoc committee under Dr. Robert McDivitt (University of Utah) to review breast cancer pathology from 27 Demonstration Detection Centers supported by NCI and ACS; Dr. C.W. Berard, member of USA Lymphoma Panel (Dr. H. Pappaport, Chairman) and faculty of 10th National Tutorial on Neoplastic Hematopathology, Project Officer for Pathology Group review of non-Hodgkin's lymphomas, Regional Pathologist for the Eastern Cooperative Oncology Group; Dr. Elaine Jaffe, faculty member of Tutorial on Neoplastic Hematopathology, faculty IAP short course on malignant lymphomas, Educational Program of the American Society of Hematology, Editorial Board American Journal of Clinical Pathology, and member Expert on Cytochemistry, International Committee for Standardization in Hematology; Dr. M.F. Stanton, member of Smoking and Health Review Board - OD, Asbestos Protocol Subcommittee (HEW), Editorial Board of Monograph Series (IARC-WHO), Associate Editor Journal of Environmental Pathology and Toxicology; Dr. E.W. Chu, faculty member of Tutorial on Clinical Cytology, consultant to collaborative project on urinary cytology for screening and early diagnosis of bladder cancer (DCT and Cairo Cancer Institute); Dr. N.A. Young, attending physician for infectious disease consultation services in the Clinical Center and National Naval Medical Hospital, faculty of Uniformed Services Medical School, and member Virus Diseases Study Section (WRAIR); Dr. L.B. Thomas, Dr. A.S. Rabson and staff, lecture series in pathology to Georgetown University medical students and pathology lectures to students of Uniformed Services Medical School; Dr. D.E. Henson, Short Course on Pathology of Virus Infections, IAP, member of official pathology delegation visiting USSR; Dr. J.C. Costa, member of national review committees on the pathology of ovarian carcinomas and melanomas; and Ms. M.C. Bowling, editorial consultant Encyclopedia and Dictionary of Laboratory Medicine and Technology. Other activities of this sort are listed under the portions of this report dealing with each of the Sections.

Three residents completed training in June, 1977. Dr. Jan Orenstein left to take a position at the George Washington University Medical School; Dr. Peter Howley and Dr. Robert Reddick both continued their research programs as Senior Staff Fellows in the Laboratory of Pathology. Three new residents, Drs. Joseph Fantone, Mark Popovsky and Cheryl Reichert, started training in pathology in July, 1977. As of June 30, 1978, Drs. William Cook, Lance Liotta and James Strauchen completed residency training. Dr. Cook has a position at the University of Alabama Medical School and Dr. Strauchen has accepted a position in the Department of Pathology at Stanford. Dr. Liotta will continue in experimental pathology in the Laboratory of Pathophysiology, NCI, under Dr. Pietro Gullino. Three new residents, Dr. David Jensen, Dr. Josef Luetzeler and Dr. Kusum Rao started pathology training July 1, 1978.

Dr. Yoshiko Nakamura was a Visiting Fellow with Dr. Young until June 15, 1978 when she returned to Japan to take a position at the Aichi Cancer Institute in Nagoya. Dr. Richard Gordon resigned from the Image Processing Unit to take a position at the University of Manitoba. Dr. Jack Sklansky spent three months with the IPU as an Expert and then returned to the University of California, San Diego in February, 1978. After working with Dr. Dawe in Comparative Oncology Section for two years, Dr. Yuji Kurokawa returned to Japan to take a position at the National Institute of Hygienic Sciences in Tokyo. Dr. Suzanne Fisher started a fellowship under Dr. Dawe in March, 1978. She will study and compare a number of isozyme systems in developing and transformed tissues and tumors in vivo and in vitro. Dr. Paul S. Dickman from the University of California, San Diego has accepted an IPA appointment and will start working with Dr. Triche in July, 1978. Dr. Bo Nilsson from Lund, Sweden, will be a Visiting Fellow starting September, 1978 and will work with Dr. David Zopf to develop radio-immunoassays to analyze complex carbohydrates in cell membranes. Dr. Robert Farnham from Duke University joined the Hematopathology staff as a Guest Worker in January, 1978 and Dr. Henry Azar, also a Guest Worker, started working in Hematopathology in July, 1978. Dr. Ming Fan Law started working with Dr. Peter Howley as a Visiting Fellow in August, 1977. Because of the reorganization for contract programs, Dr. Bill Bunnag, who has been with Dr. Herman in Quantitative Cytology, was re-assigned to the Office of the Director, DCBD, in May, 1978. Dr. Stuart Bentley started a two year assignment as an Expert with Dr. Herman in November, 1977. Dr. Suresh K. Arya accepted an IPA position with Dr. Young in October, 1977. Dr. Iwao Nishiya, from Hokkaido University School of Medicine, worked with Dr. Herman from October, 1977 to July, 1978 as a Guest Worker, and Dr. Joachim Betz was an Expert from the Battelle Institute, Frankfort, West Germany and worked with Dr. Herman from July, 1977 to December, 1977.

The staff were either senior authors or coauthors of 76 scientific papers published in 1977. Seventeen papers have been published through April, 1978 and, in addition, there are currently 49 papers in press and 30 others submitted for publication.

Surgical Pathology and Postmortem Service

Dr. José C. Costa together with Dr. Bruce L. Webber and the residents and other pathologists from the Laboratory of Pathology plus Dr. Allen W. Cheever (NIAID), Dr. Robert Friedman (NIAMDD), Dr. William C. Roberts (NHLBI) and Dr. Kurt Stromberg (NCI) were responsible for the surgical pathology and autopsy diagnostic services. 6779 surgical specimens or biopsies were accessioned in the past year (includes approximately 2300 bone marrows); 224 autopsies were accessioned (68 of these were submitted). These specimens involved the preparation of more than 65,000 slides, including more than 42,000 H&E stained sections, nearly 8000 special stains and 531 frozen sections.

Many specimens of fresh human tissues, including the eyes which are regularly removed during a complete autopsy, are furnished to NIH scientists in various laboratories.

Conferences with individual clinicians and surgeons from the various Institutes are an important part of the care and study of patients in the Clinical Center. Regular gross specimen reviews of organs removed at autopsy are conducted by the staff and most surgical specimens are discussed at the weekly Surgical Pathology Conference conducted by Dr. Costa, Dr. Webber, and the residents and staff. In addition, the staff participates in other regular conferences including Pediatric Oncology Tumor Conference (Dr. Triche), Hematopathology and Lymphoma Staging Conferences (Dr. Berard, Dr. Jaffe and staff), a monthly Neuropathology Conference (Dr. Valsamis) and conferences on Sarcoma Staging, Surgical Morbidity and Mortality, Medicine-Radiation Therapy (Dr. Costa and staff). The staff and residents present selected autopsies at monthly clinico-pathologic conferences (e.g. NCI - Dr. Costa, NHLBI - Dr. Roberts). A weekly seminar for presentation of research by the pathology staff has been organized by Drs. Herman and Howley. Weekly slide presentations by the residents using current surgical and autopsy cases are held as a part of their training program. Finally, most Section Heads have a weekly conference with their professional and technical staff to discuss ongoing programs and research projects.

Publications of Studies on Human Material

Slightly more than half of the 76 publications by the staff and residents in 1977 involved human tissues or some aspect of human disease. These included culture and study of hormone-dependent human breast cancer (Young-1); delayed hypersensitivity in Graves' disease and exophthalmus (Mullin, Levinson, Friedman and Henson-3); description of two patients with endocarditis due to Cardiobacterium hominis (Young, Horvath-4); clinical studies and general reviews of malignant lymphomas (Jaffe-2, Braylan, Jaffe, Berard-9, Reddick-31, Jaffe, Braylan, Nanba, Berard-41, Berard, Jaffe-42, Berard-56-58, Frizzera, Berard-59, Banks, Berard-61); electron microscopy study of annulate lamellae in diffuse lymphocytic lymphoma (Watanabe, Berard, Triche-10); surface receptors on neoplastic lymphoreticular cells (Braylan, Jaffe, Mann, Berard-15); parathyroid gland hyperplasia (Reddick, Costa-11); T-antigen expression in skin fibroblasts from patients with cryptogenic anomalies (Costa, Rabson-16); cellular distribution of amyloid AA protein (Watanabe, Jaffe-17); enzyme histochemical characterization and splenic stromal changes in hairy cell leukemia (Nanba, Jaffe, Soban, Braylan, Berard-18); splenic pseudosinuses and hepatic angiomatous lesions in hairy cell leukemia (Nanba, Soban, Bowling, Berard-24); compensated primary hypothyroidism in cystinosis (Howley-26); review of breast cancer cases included in HIP study (Thomas-29); terminal deoxynucleotidyl transferase in malignant lymphoma cells (Jaffe, Braylan-30); cutaneous infarction in disseminated mucormycosis (Reddick, Levine-32); relationship of prenatal phenytoin exposure to malignant mesenchymoma and birth defects (Henson-35); gastrointestinal involvement in Sezary's syndrome (Jaffe-36); absence of papovavirus T antibodies in patients with malignancy (Costa, Yee, Rabson-37); ectopic chorionic gonadotropin and alpha subunit secretion by a cultured human lung cancer (Tralka, Rabson-40); a Waldenström macroglobulin that is both a cold agglutinin and cryoglobulin (Zopf-43); alkaline phosphatase-positive malignant lymphoma (Nanba, Jaffe, Braylan, Soban, Berard-55); Burkitt's tumor (Mann, Berard-60); synthesis of procollagen in cultured cell line of glioblastoma multiforme (Rabson, Stern, Tralka, Costa-62); epidemiologic, etiologic and pathologic studies on hepatic angiosarcoma (Thomas-63,66); prognostic factors in patients with lymphadenectomy for malignant melanoma (Tomaszewski, Costa, Rabson-64); epidemiologic study of cancer of cervix, vagina and vulva (Henson-67); prosthetic valve endocarditis (Garvin, Roberts-69); red cell survival (Bentley-71) and fine needle aspiration cytology in diagnosis of thyroid nodules (Chu, Hanson-76).

There are a large number of other ongoing studies of human disease which are listed in individual annual reports. A list of some of them includes: evaluation of carcinoma in-situ of the breast in Connecticut and California by Drs. Costa and Webber; review of breast cancer cases in Tunisia by Drs. O'Conor, Webber, and Costa; review of testicular tumors by Drs. Popovsky and Webber; review of skin tumors in patients with Xeroderma pigmentosum by Drs. Popovsky, Webber, and Costa; immunohistochemical study of basement membrane collagen in differential diagnosis of lymph node metastases by Dr. Liotta and Miss Bowling; study of cytologic characteristics of cell types seen only in fine needle aspirations by Dr. Chu and staff; comprehensive EM study of the ultrastructure of various types of liposarcoma, osteogenic sarcoma and other soft tissue sarcomas by Drs. Reddick, Triche, and Levine; completion of epidemiologic and pathologic studies on hepatic angiosarcoma by known etiologic agents (vinyl chloride, thorotrast, and arsenic) and due to unknown causes by Drs. Thomas, H. Popper, N. Telles, and H. Falk; review of some 600 non-Hodgkin's malignant lymphoma cases in the files of the Laboratory of Pathology by Drs. Garvin and Berard; continuing efforts to detect evidence of RNA tumor virus in cultured cells of human breast carcinoma by Dr. Young and Dr. Arya; study of myosins in soft tissue sarcomas by Dr. Henson and Ms. Levinson; and participation in a national study of ovarian cancer by Dr. Costa (Reference Center is under Dr. S. Gallagher, M.D. Anderson Hospital).

Dr. Costa and Dr. Rabson, together with Dr. Y. Nakamura, a Visiting Fellow, Dr. U. Thorgeirsson from George Washington University, and Ms. Yee and Ms. Tralka, have continued their experimental studies of human herpesviruses and papovaviruses. The topology of the HSV induced Fc receptor has been determined by immunofluorescence and it appears to be admixed with other viral antigens. It appears possible to co-immunoprecipitate viral induced proteins with antigen-antibody complexes. The binding of viral protein is saturable and is dependent on the presence of the Fc region of the antibody molecule. Further studies to characterize the biochemical structure of the Fc binding molecules have been started. In other experiments it has been shown that bound IgG and immune complexes internalize. The biological properties of papovaviruses (K virus, SV40 and SV40 mutants) and their DNA in vivo and in vitro are being studied. The proposed course of these various studies can be summarized as follows: 1. Binding of immunoglobulin and internalization will be quantitated. 2. The effect of IgG on HSV DNA synthesis in different strains will be compared. 3. The effect of HSV infection upon the cell membrane will be studied using fluorescent membrane labeling procedures developed in our laboratory in collaboration with Dr. Zopf and Dr. Stewart (NIAMDD). 4. The effect of EBV infection on Fc receptors will be studied in collaboration with Dr. G. Klein.

Miss Bowling and her staff continue to provide excellent histological slides and special stains for human tissues. She is also a valuable consultant on histological techniques to numerous NIH scientists and Clinical Center physicians. The handling of human tissues is highly integrated with the performance of autopsies and the use of human tissues for special studies. The autopsy assistants, Mr. Herndon, Mr. Dock and Mr. Mattison, play a major role in assisting residents in the actual performance of the autopsies and in the fixing, distribution and storage of human specimens.

Photographic services for human tissues and experimental studies are provided by Mr. K. Edwards, Mr. Banks and Mr. Isenburg. Mr. Banks also maintains the slide files which now number over a million slides of human tissues accumulated since the Clinical Center opened in 1953. These are frequently used for comparative and retrospective studies and constitute an invaluable pathology archive of human diseases studied at the Clinical Center. Mr. Ralph Isenburg and Mr. Banks not only provide a large proportion of photomicrography and other special photographic services for the staff, but they also maintain a photographic laboratory which is used by numerous Laboratory of Pathology investigators and technologists, especially by those who develop and process EM photomicrographs. Photographic documentation is essential for the study of pathologic changes in all organs, especially for the cardiovascular system. A reflection of this fact is that Mr. Edwards and Mr. Banks took more than 2000 black and white, 4 x 5 inch gross pictures and more than 11,000 color photographs of surgical and autopsy specimens between April, 1977 and March, 1978.

Cytopathology Section

Dr. Chu and her staff provide diagnostic services in exfoliative cytology, medical genetics and fine needle aspiration. Chromosomal analysis includes standard karyotyping and special banding techniques for the examination of individual chromosome pairs. During the year approximately 3,800 cytology specimens were accessioned. These represent over 9,000 smears which were examined and reported. A little less than 1/4 were cervical and vaginal specimens; 3/4 were other types of specimens including ascitic, pleural, gastric, prostatic and spinal fluids, bronchial specimens, wound washings and fine needle aspirations. In addition to the diagnostic reports, Dr. Chu and her staff collaborate with the clinical and surgical staff in various research projects to evaluate cancer therapy and the hormonal status of the cancer patient and to follow the course of cancer in particular patients. Specific examples of these are the cytologic evaluation of degrees of relapse or remission during the course of chemotherapy of meningeal leukemia and evaluating the therapeutic effects on carcinoma of the urinary bladder by studying the cytology of repeated, sequential urine specimens. During the past year Dr. Chu and Mr. DelVecchio have each visited the Cairo Cancer Institute, Cairo, Egypt to provide professional and technical support for a collaborative project supported by OIA and DCT which is designed to evaluate urine cytology as a screening method for the early diagnosis of bladder cancer. Cytological methods are also used in support of collaborative research with other scientists in the NCI.

Quantitative Cytology Section

Dr. Herman and his staff including Ms. Habbersett, Dr. Alabaster, Dr. Bunnag, Dr. Bentley and Guest Workers Dr. Betz and Dr. Nishiya are responsible for intramural research using cell sorters and flow microfluorometry (FMF) techniques. They have several major projects. Among them is the use of cytochemical stains (propidium iodide and fluorescein isocyanate) to analyze cervico-vaginal cytology specimens to automate the cytological diagnosis of cervical cancer. During the past year, two methods were used to analyze quantitatively the data from FMF experiments. One of these (Ms. Habbersett and Mr. Shapiro) involved use of a computer based decision tree logic program. In a double blind study 80.2% of 124 cytology specimens were analyzed correctly with a false positive rate of 27% and a false negative rate of 9.6%. Specimen adequacy was also evaluated quantitatively and a machine calibration and a staining standard were developed. In a second experiment (Dr. Bentley, Mr. Smith and Ms. Habbersett) Fourier transform analysis was used to evaluate data from the same set of cytological specimens. 79.6% were correctly diagnosed in two different test sets; the false positive rate was about 15% and the false negative rate was about 30%. Thus both of these methods hold promise for use in automating cytologic screening of cytology specimens and work will continue to improve the biological resolution of these techniques.

In experiments conducted principally by Dr. Braylan (Hematopathology Section) and Ms. Fowlkes, sixty-five normal and neoplastic human lymphoid cell specimens were analyzed by FMF for DNA content. For neoplastic cells, they found a general correlation between the clinical progress of the disease and the relative sizes of their S phase populations. FMF techniques were used to compare the cytokinetic response of P388 leukemic ascites cells exposed to Maytansine and vincristine. The responses to these chemotherapeutic drugs were similar (Dr. Alabaster and Ms. Habbersett).

A significant project (Mr. Tannenbaum, Ms. Habbersett and Dr. Alabaster) was successfully completed during the year. This involved using chicken erythrocytes, as an internal biological staining standard, for the analysis of DNA content of normal and neoplastic cells studied with FMF techniques. After methods of preparation and preservation of the standard were worked out, studies of DNA fluorescence intensity changes in drug perturbed L1210 ascites cells were made. The drug treated cells showed changes in fluorescent intensity relative to the standard which were not seen in controls. Thus use of chicken erythrocytes as an internal biological standard appears to be a valuable tool in the analysis of cell cycle kinetics. These and other FMF methods will continue to be actively used to study cell kinetics of neoplastic cells from leukemia and lymphoma patients who are currently undergoing chemotherapy.

Several projects were started to attempt to measure the histone content of normal and transformed cells by FMF techniques. Eosin Y as a fluorescent dye for histone proved unsuitable. Attempts to develop a fluorescenated anti-H-1 complex for immunologic tagging of the H-1 histone fraction seem promising; Dr. Betz will continue this work after returning to the Battelle Institute. Dr. Nishiya's ongoing projects involve the study of the effects of hormones (DES, estradiol, estriol, progesterone, testosterone, prostaglandin E and F) on the cell cycle of HeLa cells using FMF and the internal chicken red cell standard. Dr. Herman was awarded an assignment by the Japanese Government Research Awards for Foreign Specialists for a 6 month period from May to December, 1978. He will spend this time working in the laboratory of Dr. Yoshio Uei at the National Cancer Center Hospital in Tokyo, Japan.

Ultrastructural Pathology Section

Dr. Triche and the staff in the Ultrastructural Pathology Section are responsible for diagnostic EM and for a number of research programs using electron microscopy and freeze-etching techniques. In the past year, the first in the newly renovated and relocated EM laboratory, Dr. Triche, Dr. Reddick, and staff have undertaken new major research commitments, previously impossible in the former laboratory, while continuing to provide a diagnostic EM service. Further, Dr. Zopf and Dr. Garvin have likewise commenced work in their newly completed laboratories.

The diagnostic EM service has functioned better than at any time in the past, due in large part to Dr. Reddick's unflagging efforts and in part to the close proximity to the surgical pathology laboratories. From January, 1977 to January, 1978, 234 specimens were processed for EM; of these, 128 were processed to completion. Of these, 52 were clearly of diagnostic relevance and served to improve or change the light microscopic diagnosis. From January, 1978 through June, 1978, 148 specimens have been processed, further expanding the availability and use of the diagnostic EM service. Three residents received some EM training during the year, but the lack of instrument time prevented development of a more complete training program. This program will be improved when a second instrument, the Philips EM 201, purchased sometime ago for the anticipated diagnostic EM facility, is placed into operation in 2A09. It will provide adequate instrumentation for diagnostic and training purposes, as well as furnishing more EM scope time for the research of Dr. Garvin and other staff.

The EM 400 is scheduled to be modified with the addition of a high resolution scanning attachment this summer, thereby completing its capabilities, which now include advanced EM technology for examination of entire cells grown in tissue culture, high resolution negative staining and replica examination, and, with the addition of the scanning attachment, cell surface studies at very high resolution, in conjunction with simultaneous cell interior correlative study. This should allow identification of specific membrane molecules on normal and tumor cells for correlation with biochemical data derived from cell labelling and solubilization studies by electrophoresis and isoelectric focussing. The major new research commitment in the laboratory is the morphological and biochemical identification and characterization of cell membrane proteins and complex carbohydrates, which appear to be altered or absent in tumor cells, in an attempt to correlate aggressive biological behavior with membrane components. Much animal model work has focussed on a 220,000 molecular weight glycoprotein (LETS) which is consistently absent or diminished in animal tumors; preliminary results with the cell lines derived from patients at the Clinical Center in the past year are at variance with these data. Conflicting and supportive data on other human tumors *in vitro* have also appeared, somewhat confusing the true situation in humans. Further study will obviously be necessary, and will include consideration of other proteins, as well as examination of several other, better characterized human lines. Even if such proteins are biochemically intact, morphological studies have suggested an abnormal distribution and mobility of such proteins in animal tumors; similar defects may be detected in human tumors by use of the advanced morphologic techniques outlined above.

Collaborative studies with other investigators have been completed in some cases, and continue in others. Lymphoma studies have been completed with Dr. Braylan and Dr. Watanabe, and are nearly complete with Dr. Nanba (the principal investigator in each case). A short project on the effects of Adriamycin therapy on wound healing with Dr. Devereux in Surgery Branch is complete, as is a collaborative study with Dr. Mukharje in Child Health and Human Development on liposome-mediated transfer of genetic information to acceptor cells. Work on the identification of isolated, purified IgE Fc receptor from basophils continues with Dr. Henry Metzger, as does the second phase of a study on the biological fate of basophil-bound IgE. The first phase has just been completed and is being submitted for publication.

Dr. Triche has agreed to write two chapters (the second in collaboration with Dr. David Poplack) for a forthcoming monograph on pediatric oncology, edited by Dr. Art Levine of the Pediatric Oncology Branch, NCI. This is a logical outgrowth of the continued involvement by Dr. Triche, as well as by Dr. Reddick and Dr. Garvin, in the POB tumor board.

Dr. Reddick and Dr. Levine have had a scientifically productive year; collaborative work on osteosarcoma cells in vivo and in vitro has resulted in publications with Dr. Triche and Dr. Steve Rosenberg. Studies of soft tissue sarcomas have resulted in the preparation of several manuscripts.

A brief description of Dr. Garvin's review of non-Hodgkin's lymphoma cases is included in the Hematopathology report. His laboratory research includes continuing study of the mechanism by which lysosomal enzymes are secreted and then adsorbed by normal human fibroblasts. Two populations of lysosomes have been identified by biochemical and ultrastructural histochemical methods and this is a significant advance in the understanding of the function of the Golgic apparatus and the lysosomal secretion and adsorption system. These studies may lead to a better understanding of the pathogenesis of some genetic diseases and of rheumatoid arthritis. Dr. Garvin has also started experiments to isolate, purify and identify the HY antigen which is believed to be involved in sexual differentiation of the gonads. This work, in mice, may lead to a better understanding of the mechanisms of aberrant development which sometimes leads to dysgenesis and to the development of neoplasms.

Dr. Zopf spent part of the year establishing his laboratory in order to continue his research on a variety of oligosaccharides and other complex carbohydrates produced by cells. Specifically, he developed a radioimmunoassay technique to determine accurately an Le^b-active oligosaccharide in urine. By using this method to study the urine of 138 pregnant or lactating women with different Lewis blood types, he showed that excretion of this oligosaccharide reaches a maximum in the third trimester and continues through the postpartum period during lactation. The evidence suggests that the source of this oligosaccharide may be the mammary gland and its secretion quantitatively is correlated with specific blood types. Other oligosaccharides are being studied by these techniques in ongoing experiments. In other experiments, Dr. Zopf studied the immune responsiveness of CBA/N mice (an inbred strain that exhibits an X-linked deficiency in immune responsiveness to certain carbohydrate antigens including dextran to isomaltosyl oligosaccharides coupled to protein carriers. His experiments showed that antibodies that bind dextran can be elicited by immunization with isomaltotriosyl-protein carrier (keyhole-limpet hemocyanin) in CBA/NX Balb F_1 males and females and in normal Balb mice and that the immunodeficiency to polysaccharides of CBA/N mice can be circumvented when the polysaccharide determinants are presented via a "T cell dependent" route. These findings open up the possibility of vaccination against bacterial organisms (e.g. pneumococcus), which in their normal state, are poorly immunogenic. A major new research project involves developing and using methods for fluorescence labelling of surface galactose residues. Cells treated with galactose oxidase and stained with Lucifer yellow carbohydrazide have a brilliant yellow fluorescence and remain viable for a number of days. Thus they can be observed to follow migration of the tagged surface glycoconjugates. This method will contribute to an understanding of the biological fate of these glycoproteins and may help determine whether certain viral glycoproteins appear as host cell components prior to their incorporation into the virus particles.

Dr. Donald Henson was assigned to the NCI Office of International Affairs in October, 1977. He continued, on a part-time basis, experiments utilizing immunofluorescence and EM techniques to study cell receptors and cell products in a variety of normal and neoplastic human and animal tissues. Included were surface binding sites for cholera toxin, TSH, myosin in rhabdomyosarcomas and other soft tissue sarcomas and osteosarcomas, and reactivation of chronic CMV infection in mice by immunosuppression and anti-lymphocyte serum. These experiments potentially could aid in a more accurate diagnosis of certain neoplasms and in some understanding of the functional capacities of neoplastic and undifferentiated cells. Dr. Henson also continued with his review of the pathological lesions occurring among members of families found to have a high incidence of cancer. These studies are in collaboration with Dr. J. Fraumeni and his staff in the Environmental Epidemiology Branch, NCI.

Hematopathology Section

Drs. Berard, Jaffe, Cossman, Callihan and their guest worker, Dr. Robert Farnham from Duke University, have major programs in diagnostic and experimental studies on neoplastic and non-neoplastic lesions of the lymphoreticular and hematopoietic systems. Dr. Berard has a large number of referral cases of malignant lymphomas including 230 cases from the Eastern Cooperative Oncology Group submitted to him as Regional Pathologist for the Pathology Panel for Lymphoma Clinical Trials and 583 cases submitted for diagnostic consultation from pathologists in the United States and foreign countries. In addition, he and his staff are responsible for the diagnostic reports and staging conferences on cases of malignant lymphoma admitted to various clinical services in the Clinical Center. Fresh biopsy tissues have been frequently available from Clinical Center cases and during the past year more than 120 such specimens were used by Drs. Jaffe, Cossman, Callihan and Farnham for special immunologic, histochemical, immunocytochemical, and ultrastructural studies. This is a very productive program as indicated by the relatively prolific number of publications (see attached list of publications). Included were studies on the immunologic, histochemical, morphologic, and kinetic features of cases of non-Hodgkin's lymphomas of all subtypes, hairy cell leukemia, non-endemic ("American") Burkitt's tumor, alkaline phosphatase-positive malignant lymphoma (a subtype of B-cell lymphomas), and acute lymphoblastic leukemia. Clinico-pathologic studies were published on nodular sclerosing Hodgkin's disease, lymphomas obscured by concomitant extensive epithelioid granulomas, advanced lymphocytic lymphomas randomized to chemotherapy and/or radiotherapy, non-Hodgkin's lymphomas treated by combination chemotherapy, and survivals of nodular versus diffuse poorly differentiated lymphocytic lymphomas.

Drs. Berard and Jaffe were each invited to give several lectures and/or seminars at scientific meetings and continue to serve as faculty members for the Tutorials on Neoplastic Hematopathology organized by the Center for Continuing Education of the University of Chicago under the scientific direction of Dr. Henry Rappaport. These educational efforts have been ongoing for several years and briefly mentioned in previous annual reports but their importance has not been specifically stressed. The developments in the diagnosis and clarification of the malignant lymphomas have been particularly dynamic during the past several years because of rapidly increasing knowledge about the functional characteristics of the normal and neoplastic cells of the lymphoreticular and hematopoietic systems. Dr. Berard, Dr. Jaffe and their associates have made major contributions to this increased knowledge and also have participated in many national and international meetings, symposia, and tutorials to bring this new information to practicing pathologists, clinical hematologists-oncologists, and basic scientists. They have served as faculty members at the 10th and 11th Tutorials on Neoplastic Hematopathology, conducted respectively at Pasadena, California on October 10-14, 1977 and at the University of Chicago on February 6-10, 1978. In addition they have individually and/or collectively presented major lectures and seminars for the International Academy of Pathology (Toronto, March, 1977 and Atlanta, March, 1978), the International Colloquium on Lymphoid Neoplasias (Paris, June, 1977), the Leukemia Society of Rhode Island (Providence, September, 1977), the American Cancer Society-National Cancer Institute National Conference on the Lymphomas and the Leukemias (New York City, September, 1977), the annual meeting of the Eastern Cooperative Oncology Group (Chicago, November, 1977), the fifth annual meeting of the Australian Division of the International Academy of Pathology (Sydney, Australia, May 27-28, 1978), the Universities of Sydney, Melbourne, Adelaide, Perth and Singapore, the University of Maryland, George Washington University, Tufts University, the University of Minnesota, Hospital Henri Mondor (Creteil, France) and the annual meeting of the American Society of Hematology. This sample of their recent presentations is cited in order to stress the importance and magnitude of their teaching efforts nationally and internationally and the impact that these efforts have had on the field of neoplastic hematopathology.

Drs. Berard, Jaffe, Callihan and Cossman participate fully in other activities of the Laboratory of Pathology including the teaching of residents and the preparation of weekly Oncology (Thursday morning) and Lymphoma Staging (Friday afternoon) conferences. Dr. Jaffe is chairman of the departmental residency selection program. Dr. Berard serves as project officer for five large-scale contracts awarded by NCI's Division of Cancer Treatment to evaluate newly proffered classifications of non-Hodgkin's lymphomas. These contracts involve clinico-pathologic review of 1175 cases at four institutions by a panel of 12 expert hematopathologists, of which Dr. Berard is also a member. In addition, Dr. Berard is a member of an Ad Hoc Pathology Working Group organized to advise the Division of Cancer Treatment on mechanisms and policies for pathologic review of cases entered into clinical therapeutic trials.

Laboratory of Pathology, DCBD, NCI

Comparative Oncology Section

Drs. Dawe, Banfield and Stanton are the professional staff members of this Section. Dr. Dawe has continued his studies of epithelial-mesenchymal (stromal) interactions and their relationships to tissue growth, differentiation, hyperplasia and neoplastic transformation. The principal findings are that neoplastic changes induced in mouse salivary gland epithelium by polyoma virus have widely variable characteristics depending upon whether the transformational and post-transformational events take place in the presence or absence of stromal cells. The evidence suggests that stroma may act to inhibit or decrease tumor progression and to influence the type and degree of differentiation expressed by the neoplastic cells. For example, neoplasms from salivary gland epithelium transformed *in vitro* by polyoma virus (PV) in the absence of stroma were completely undifferentiated. Neoplasms induced by PV in the presence of stroma showed some differentiation into glandular, squamous or bone and cartilage forming components. Also the *in vitro* transformed neoplastic cells had many aneuploid cells; *in vivo* transformed cells were diploid or near-diploid. Finally, *in vitro* transformed tumors when transplanted to syngeneic mice would frequently metastasize whereas only rare metastases occurred from transplanted *in vivo* transformed tumors. In another set of experiments, *in vitro* tumors transformed in the absence of mesenchyme would cause only a minimal degree of morphogenesis of normal salivary gland epithelium whereas tumors produced *in vivo* would interact with normal salivary gland epithelium to produce extensive degrees of morphogenic development (branching, duct and gland formation). These experimental models have been used in numerous other ways; e.g. to test the interactions of epithelium with stromal tissue from skin and dental anlage. Obviously there could be major significance in the experimental demonstration that the same oncogenic virus (PV) acting on the same target cells (salivary gland epithelium) can induce morphologically and biologically different neoplasms if the microenvironmental conditions are varied during and/or after the transformational events. It is suggested that the primary transformational events are the same, regardless of microenvironmental variables, but these same variables may modify subsequent tumor progression. It would be useful to find the mechanism of this phenomenon so that tumors *in vivo* could be influenced to undergo differentiation and deceleration of growth.

Dr. Suzanne Fisher has started experiments to isolate and compare isozyme systems in the PV tumors induced *in vivo* with those produced *in vitro*. In other experiments Dr. Jules Berman is collaborating with Dr. Dawe to study collagen production in established culture lines of hepatomas and in PV-transformed salivary epithelium. These various experiments are designed to determine some of the functional characteristics of the various tumors and hopefully lead to an identification of one or more factors in PV tumors or stroma which are responsible for the observed effects on the growth and morphogenesis of these tumors.

Mr. Morgan's research projects using time-lapse photomicrography have continued and have been used to study the 3 types of cell contraction previously observed in organ cultures of mouse submandibular gland rudiments.

Dr. Dawe and his staff continue to work with Dr. Harshbarger and the staff of the Registry of Tumors in Lower Vertebrates and Invertebrates to study certain phylogenetic aspects of neoplasia. When appropriate fresh tissue is available, efforts are made to establish cultured tumor cell lines. Currently there are cultures of a corn snake chondrosarcoma (continuously cultured for 7 1/2 years), a trout nephroblastoma (5 years) and a corn snake tumor which is possibly a liposarcoma or chromatophoroma (1 month). These cultured cells are being used for a variety of experiments; e.g. to determine whether they maintain tumorigenic properties, whether they maintain capacity to differentiate, whether viruses are present in the tumor cells and whether it is possible to produce immunologic responses against these tumors in mammalian hosts.

During the year the ETEC scanning electron microscope - electron probe equipment was moved from the 8th floor laboratory into NIAMDD space in Building 6. There is now a cooperative arrangement between Dr. Banfield and Dr. Hagins to use this equipment. The plans are to purchase a wave length dispersive spectrometer and other equipment so that biological materials can be examined by SEM, light microscopy and x-ray analysis. Dr. Banfield is continuing with his study of elastin in specimens of skin from patients with senile elastosis and of stratified squamous epithelium. Also Dr. Banfield is continuing collaborative studies of the lamella-particle complex seen in a number of tumor cells and virus infected cells.

Dr. Stanton's principal research for the past several years has been pulmonary and pleural carcinogenesis using several animal models which he developed. These have been described in previous annual reports. The chief carcinogenic agents being studied with the beeswax pellet method are petroleum-soluble air pollutants, tobacco smoke condensates, and asbestos and other fibrous particles. In these experiments, some 4,000 rats have received different fractions of tobacco smoke condensates. These condensate fractions were obtained and are being studied in collaboration with investigators from the Agriculture Research Center in Athens, Georgia. Results to date show the PAH fraction is the most carcinogenic and that acidic fractions and methanol subfractions are also carcinogenic. The significance is that removal of any of these fractions from cigarette smoke might reduce pulmonary carcinogenicity. Similarly PAH crude extract of air particulates (obtained from EPA) is also carcinogenic for the lung and refined subfractions of these extracts are now being tested.

In previously reported experiments Dr. Stanton has shown that durability and fiber size (<0.25 µm diameter x >8 µm length) of asbestos and fibrous glass were critical to the production of pleural and pulmonary neoplasms in rats. Pleural neoplasms have now been produced in another species (BALB/c mice) exposed to asbestos and fibrous glass particles. The data, from experiments still to be completed, suggest that fibrous particulates less than 0.02 µm and those with a diameter of 3 µm or larger are not carcinogenic. Obtaining and working with particulates of uniform, appropriate size is technically most difficult and these studies are being continued to elucidate further the critical nature of particulate size and shape in carcinogenesis. During the past year, Dr. Stanton has participated in studies of a number of tumors which occur in Osborne-Mendel rats. These tumors were collected in this laboratory over the years from the autopsies of more than 4,000 aged females of this strain. Of particular interest in this review are a type of lipo-reticular sarcoma of the kidney and also a variety of pulmonary tumors, osteogenic sarcomas and mammary gland tumors. Studies of these collected tumors will be useful in establishing incidence rates of spontaneous neoplasms in O-M female rats and for comparison of these rates with experimentally induced tumors.

Viral Oncology and Molecular Pathology Section

Dr. Young has continued his research on the polypeptide markers in various strains of vaccine and wild type polioviruses.

The relevance of the polypeptide marker (see previous annual report) to neurovirulence of type 1 poliovirus was assessed by the following experiments. Two clones of virus were isolated from the stool 14 days after feeding LSc2ab vaccine to a normal child. One clone had an unstable VP1 (designated VP1 minus) while the other was representative of the mutant vaccine virus with stable VP1 (designated VP1 plus mutant). Each clone was inoculated into 29 rhesus monkeys. The VP1 minus clone produced paralysis in 6 monkeys with a histologic severity ratio of 8/29 and spread ratio of 5/29. The VP1 plus mutant paralyzed 9 monkeys with a severity ratio of 11/29 and a spread ratio of 5/29. These results indicate that both the VP1 plus and VP1 minus clones isolated 14 days after vaccine feeding are significantly ($p = 0.000018$) and ($p = 0.003$) respectively, more neurovirulent than reference LSc2ab vaccine poliovirus but not significantly different from each other. We conclude that the polypeptide marker is not an absolute correlate of the presence or absence of neurovirulence. Further studies are in progress to define the relationship of the polypeptide marker to previously known markers, such as rct 40 and the antigenic marker (Wecker analysis).

Preliminary work with polypeptide profiles of type 2 poliovirus strains indicate that it is not possible to reliably differentiate the Sabin vaccine strain from all wild strains. However, virus strains recovered from the gut and central nervous system of a case of vaccine-associated paralytic disease in an immunodeficient child had polypeptide profiles which differed from reference type 2 poliovirus vaccine. These results are consistent with the hypothesis that vaccine mutations rather than host factors alone may be responsible for the paralysis.

Drs. Young, Strauchen and Arya have continued their efforts to detect evidence of RNA tumor virus in cultured human breast cancer cells. During the past year they focused on a search for nucleotide sequences in these cells related to the genomes of mouse mammary tumor virus and Mason Pfizer monkey virus. Technical difficulties prevented significant progress.

Dr. Howley with a Visiting Fellow, Dr. M.F. Law, and in collaboration with Dr. K.K. Takemoto has continued his experiments with the murine papovavirus K. The objective is to evaluate the virus biologically in tissue culture, characterize the disease it produces in mice and characterize the nucleic acid of this virus and compare it to other known papovaviruses. Two of these, JC and SV40, are associated with progressive, multifocal leukoencephalopathy in man and monkeys and the papovaviruses are oncogenic in animals. Using the techniques of restriction endonuclease analysis, hybridization and immunofluorescence, a physical map of the K virus genome has been constructed and mapped regions of the genome conserved between K virus and PV and between K virus and primate papovaviruses have been determined. These studies are being continued so that comparative evaluation between the various papovaviruses can be completed. Dr. Howley and Dr. Law are using similar research methods to study the human papovaviruses BK and JC, as well as other primate papovaviruses SV40, SA12 and STMV.

Image Processing Unit

At the end of the previous reporting period, the unit was reviewed by a group of distinguished, biologically oriented computer scientists. The major thrust of the unit's activity during the present period has been directed toward a positive and constructive response to the review committee's major findings. These recommendations fell into essentially two major categories: i.e. 1) strengthening the biological relevance of the IPU program by intensifying and elaborating the collaborative work with biological scientists doing research at the forefronts of biomedical research, as well as their own biological work; and 2) redirecting software and hardware efforts so as to move a) toward diminished dependence on DCRT's PDP10, while b) planning to supersede obsolescent equipment by specifying (for subsequent procurement) an appropriate dedicated computer with adequate facilities for time shared image processing. Despite the enforced halt for several months occasioned by the physical move to the Park Bldg., at the end of the reporting period, gratifying achievements in the area of the first recommendation were being submitted for publication, while a significant start had been made in both reduction of PDP10 usage and dependency and in specification of a PDP11 type machine to serve the central processor needs.

Collaborative work with Dr. Jacob Maizel (Child Health) in areas of nucleic acid biochemistry has demonstrated several significant and productive roles for image processing in data acquisition, analysis and representation. Initial work, based on the need to construct secondary structure maps from electron micrographs of nucleic acid molecules is well advanced toward the goal of automated measurements of single molecular images sufficient to produce such maps. An initial publication dealing with the use of notch filtering technics for noise reduction, as applied to adenovirus images is in press, while reports on other noise reduction technics, gap filling, and background artefact removal are almost ready for submission. The actual generation of 2° structure maps from preprocessed images of non-looped, non-branched SV40 virus images has been accomplished. It is being applied to a data base which will consist of 50 or more molecular images now being acquired. A more sophisticated boundary follower would greatly aid production work here as well as in the case of almost every cellular image problem. Development of such a set of programs will proceed in parallel with the processing of molecular images, and a later resumption of the work on macrophages. The circle transform and a series of other shape descriptive procedures were applied, amplified and extended as a result of this productive biological-computer science collaboration. The algorithms as well as preliminary results on adenovirus images, form the basis of Bruce Shapiro's doctoral dissertation (University of Maryland, August, 1978).

The Maizel collaboration has served as a paradigm for selecting other image analysis problems in molecular biology. There are of the order of a dozen laboratories at NIH employing some form of 2D electrophoresis involving iso-electric focusing in one dimension. Dr. Carl Merril (NIMH), working with Drs. Ira Pastan and Max Gottesman (NCI, DCBD), presented us with radioautographs (C14) of gels applying his 20 technics to several E. coli forms including IS-2 incorporated and modified IS-2 as well as a strain without the insertion. Image processing technics developed by Peter Lemkin in his work on marrow cells (Ph.D. dissertation, University of Maryland, April, 1978) was applied to the analytical comparison of these gels. The proteins occasioned by the IS-2 insertion as well as those by the modified IS-2 were rapidly located in the image comprising several hundred protein entities. The ultimate utility of the generality of technics developed for cell analysis is thus justified in this specifically unanticipated, exciting application. The technics which rapidly located several "new" proteins among hundreds are equally appreciable to fluorescent labeled or stained material, to 2D electrophoretograms on other bases, and to technics such as those employed by Ernst Freese - chromatography and electrophoresis. Since with appropriate standards we can perform useful densitometry, the use of IPU programs and hardware for quantitation of some CNS proteins is of interest to Neurology's Laboratory of Molecular Biology.

The collaboration with Dr. Maizel's group is continuing. Following the development of some string processing programs for elucidation of patterns of base sequences, it became apparent that it might be possible to automate the task of base sequencing by direct computerized reading of the "multichannel" electrophoretograms which represent the ordering of bases in a nucleic acid molecule. Work on this is progressing rapidly, and here again, technics developed for cell analysis were immediately applicable to this problem with striking results. This, the string processing program, and the work on secondary structure are three major components which together go a long way toward an integrated package for nucleic acid biochemical analysis.

The grain counter, in addition to use for autoradiographic analysis (especially on macrophages), has been shown to be suitable for single cell enzyme kinetics. A report, concerning phosphatases and lipases, is in press and further studies on other cell types and enzymes are in progress.

A totally unanticipated use of the grain counter facility is seen in the collaborative work with Dr. Richard Croissant of Hematology Branch, NHLBI. Here the data which will allow a decision as to the nature of the mode of erythroporetin mediation of the switch from one form of sheep hemoglobin synthesis to another is being acquired. The grain counter is the only existing instrument which will allow a sufficient body of data in a reasonable time to be obtained.

Efforts to automate macrophage counts, as part of our cytotoxicity tests for asbestos, must await the development of the more powerful boundary follower mentioned above. This is particularly necessary for differential interference images. An early result on the cytotoxic effects of known sarcoma producing fibers (provided by Dr. Stanton, NCI) is puzzling in that it suggests cytotoxicity and tumor formation may not be closely correlated.

Dr. Jack Sklansky of the University of California, Irvine, spent a part of his sabbatical year in the IPU. The interaction was extremely fruitful, with a bidirectional exchange of technics. He has helped greatly in development of some of the shape descriptors noted above.

Pathological Technology Section

Part of the technology staff, under Mrs. Barbara Coolidge, is located in Building 37 and part of the staff, under Mrs. Ruby Howard, is in Frederick, Maryland. They provide histological sections and special stains for animal tissues for scientists in DCBD and in other divisions of the NCI. During FY '78 they processed more than 28,000 pieces of animal tissue, cut and stained more than 29,000 H&E slides and prepared over 4,700 special stains. Other animal tissues were accepted in their laboratory and sent out to private contractors for routine sectioning and staining. There were a total of 107 NCI investigators who submitted material for sectioning. In addition, a number of specimens were submitted for embedding and sectioning for electron microscopy. The quality and performance of the Pathological Technology staff continues to be outstanding and they represent a major technical resource for the research of scientists in the NCI.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00853-25 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Surgical Pathology and Postmortem Section

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.C. Costa	Head, Surgical Pathology and Postmortem Section (Expert)	LP NCI
OTHER:	B.L. Webber	Visiting Scientist	LP NCI

(See next page for additional staff)

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology and Postmortem Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
20	20	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Surgical Pathology and Postmortem Section, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all Institutes in those investigations which involve the use and study of human pathological material. The Staff of the Surgical Pathology and Postmortem Section also carries on independent and collaborative research.

Project Description

Staff:

Dr. Louis B. Thomas, Chief, Laboratory of Pathology
Dr. José C. Costa, Head, Surgical Pathology and Postmortem Section
+Dr. William J. Cook
+Dr. Joseph C. Fantone, III
+Dr. Lance A. Liotta
+Dr. Mark A. Popovsky
+Dr. Cheryl M. Reichert
+Dr. James A. Strauchen
Dr. Chester J. Herman, Head, Quantitative Cytology Section
Dr. Costan W. Berard, Head, Hematopathology Section
Dr. Elaine S. Jaffe
Dr. Timothy J. Triche, Head, Ultrastructural Pathology Section
Dr. Donald E. Henson
Dr. Robert L. Reddick
Dr. Clyde J. Dawe, Head, Comparative Oncology Section
Dr. Nathaniel A. Young, Head, Viral Oncology and Molecular Pathology Section
Dr. Peter M. Howley
*Dr. Allen W. Cheever, National Institute of Allergy and Infectious Diseases
*Dr. Robert M. Friedman, National Institute of Arthritis, Metabolism, and Digestive Diseases
*Dr. William C. Roberts, Chief, Laboratory of Pathology, National Heart, Lung, and Blood Institute
*Dr. Marius Valsamis, Associate Professor of Pathology, New York Medical College, Center for Aging and Chronic Diseases

The objectives of the Surgical Pathology and Postmortem Section are: (1) to provide diagnostic services in pathologic anatomy; (2) to carry out independent research; (3) to conduct a residency training program in pathologic anatomy; and (4) to collaborate with National Institutes of Health investigators in research involving use and study of human material.

+These physicians are full time Residents in the Laboratory of Pathology
*These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Section.

1. The service functions of the section during the past year included:

(a) 224 (156 "in house", 68 submitted) autopsy examinations. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held by the staff and residents each morning at 8:30. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

(b) 6779 specimens were accessioned in the Surgical Pathology and Postmortem Section. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research projects.

(c) Histological preparation and special procedures:

These functions are carried out under the direction of Miss M.C. Bowling and a complete listing of specimens processed is given in the accompanying table.

(d) Photographic services of the department:

A photographic record is made of the large amount of the pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. L. Kenzie Edwards who also maintains the equipment and carries out much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, and are used extensively for conferences, seminars and many are prepared for publication. Listed below is a breakdown of the work performed by Mr. Edwards from April 1977 through March 1978.

Time Taking Photographs:	551 hours, 30 minutes	
Time Processing:	276 hours	
No. of Black and White Negatives: 4 x 5 inches		2,274
Color Transparencies - 4 x 5 inches - gross:	1,316	
35 mm. Transparencies - gross:	9,900	
Total	11,216	
Prints Black and White, Contract Out, 4 x 5 inches		19,162

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenburg, who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

2. Independent research. The residents and staff are engaged in the following projects:

(a) Evaluation of *in situ* breast carcinoma cases of the tumor registry units in Connecticut and California (Costa, Webber).

(b) Evaluation of the role of pathological classification in the treatment of soft tissue sarcomas (Costa, Webber).

(c) Review of the breast cancer cases from Tunisia (O'Conor, Webber, Costa).

(d) Review of all testicular tumors in the files of the laboratory (Webber, Popovsky).

(e) Review of skin tumors in *Xeroderma pigmentosum* (Popovsky, Webber, Costa).

(f) Review of lung biopsies in cancer patients with lung infiltrates (Webber, Costa).

(g) Evaluation of EMI scan techniques in the workup of bone lesions (Webber, Costa, Head-Radiology).

(h) Thin sectioning methods for routine histological work (Bowling, Costa).

(i) Immunohistochemical detection of basement membrane collagen in the differential diagnosis of lymph node metastases (Liotta, Bowling).

3. Training program. Training is achieved by direct supervision of the residents' duties by the senior staff. In addition a bi-weekly autopsy conference and a weekly surgical pathology conference are specifically designed as teaching exercises.

4. Other activities. Distribution of tissues for research. The distribution of clinical materials to laboratory scientists is now coordinated in Dr. Ziegler's office by Ms. Fran Fisher (R.N.). The staff of pathology furnishes the researchers with tissue whenever possible.

Conferences. The staff takes part in the following inter-departmental meetings:

Pediatric Oncology Tumor Board (weekly)
Sarcoma Staging Conference (weekly)
Surgical Morbidity and Mortality Conference (monthly)
Medicine-Radiation Conference (weekly)
Surgical Pathology Conference (weekly)
Lymphoma Staging Conference (weekly)

Data retrieval system. In conjunction with DCRT (Vincent, Ward), a program is now available for storing and retrieving the surgical pathology material. Coding is done following SNOMED (ICDO) nomenclature.

5. Collaborative activities.

(a) Effect of chemotherapy agents and irradiation on tensile strength of scars (Webber, Devereux).

(b) Correlation of serum markers and histological features in testicular tumors (Javadpour, Webber).

(c) Evaluation of the efficacy of different modalities of lung biopsy in the diagnosis of pulmonary infiltrates (Webber, Fly).

(d) Re-evaluation of negative lymphadenectomies in melanoma patients who recurred (Reichert, Webber, Rosenberg).

(e) Pathological characteristics of familial parathyroid hyperplasia (Marx, Thorgeirsson, Costa).

6. Proposed course.

(a) Continue to provide the services described.

(b) Increase the interaction with the clinical branches in the design and evaluation of protocols.

(c) Improve the opportunities for the resident staff to participate in teaching, conferences, and seminars, and provide elective periods to be spent accomplishing research projects with the senior staff.

(d) Implement data retrieval programs.

SURGICAL PATHOLOGY AND POSTMORTEM SECTION

May 1977 through April 1978

SURGICAL PATHOLOGY

Specimens accessioned - 6,779

Blocks of tissue cut - 13,964

Blocks of tissue recut - 1,054

Slides made - 52,503

Slides stained H&E - 33,519

Slides stained special - 6,124

Frozen sections - 531

POSTMORTEM PATHOLOGY

Autopsies performed - 156

Autopsies submitted - 68

Blocks of tissue cut - 7,398

Blocks of tissue recut - 1,109

Slides made - 13,014

Slides stained H&E - 8,745

Slides stained special - 1,829

COMBINED TOTAL

Total blocks cut - 23,525

Total slides made - 65,517

Total H&E - 42,264

Total special stains - 7,953

Publications

1. Cohen, M.H., Ketcham, A.S., Felix, E.L., Li, S.-H., Tomaszewski, M.M., Costa, J.C., Rabson, A.S., Simon, R.M., and Rosenberg, S.A.: Prognostic factors in patients undergoing lymphadenectomy for malignant melanoma. Ann. Surg. 186: 635-642, 1977.

2. Brereton, H.D., Matthews, M.M., Costa, J.C., Kent, C.H., and Johnson, R.E.: Mixed anaplastic small-cell and squamous-cell carcinoma of the lung. Ann. Intern. Med. (in press)

3. Rosenberg, S.A., Kent, H., Costa, J., Webber, B., Young, R., Chabner, B., Seipp, C., and Simon, R.: Prospective randomized evaluation of the role of limb sparing surgery and adjuvant chemoimmunotherapy in the treatment of adult soft-tissue sarcomas. Surgery (in press)

4. Kassan, S.S., Thomas, T.L., Moutsopoulos, H.M., Hoover, R., Kimberly, R.P., Budman, D.R., Costa, J.C., Decker, J.L., and Chused, T.M.: Increased risk of malignancy in sicca syndrome. JAMA (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00852-25 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.W. Chu	Head, Cytopathology Section	LP NCI
OTHER:		Medical Officer (Path.)	
	P.R. DelVecchio	Super. Res. Biologist	LP NCI
	S.D. Byrne	Res. Biologist (Cytol.)	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 6	PROFESSIONAL: 3	OTHER: 3

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Provides complete diagnostic service in exfoliative cytology, medical cytogenetics, and fine needle aspiration cytology. In addition, collaborates in various clinical research projects utilizing special techniques including special staining and tissue culture techniques.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1. To provide the staff of the Clinical Center with an accurate and complete exfoliative and aspiration cytology service as well as diagnostic cytogenetics (karyotyping).

2. To collaborate in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patients, the course and natural history of the cancer lesions and the anatomical and physiological changes in the human body associated with various pathologic conditions.

3. Development of better diagnostic techniques.

The specific objectives include:

A) Cytology

1. Continued improvement in cytologic techniques in establishing definitive diagnosis.
2. Improvement in fine needle aspiration cytologic technique.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Setting up special stain techniques which may be used for general cytology or special research problems.

B) Diagnostic Cytogenetics

1. Improvement in short term culture system to assure of successful harvesting of *in vitro* cells.
2. Improvement in staining techniques.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Setting up special stain techniques which may be used for general cytology or special research problems.

Methods Employed: 1. Special procedures in addition to the standard Papanicolaou stain are:

A) Millipore filtration of all fluids
B) Special stains:

   1. Feulgan Reaction for sex chromatin bodies
   2. Acid fast, Pneumocystis, Methenamine silver, Brown Brenn for various organisms and fungi
   3. PAS, Wright Giemsa, iron stain, melanin stain, congo-red, oil-red-0, mucicarmine for various specific reactions

2. Short term cultures of peripheral blood, bone marrow cells and tumor cells found in body fluids are the standard diagnostic methods.

In addition, special techniques involving trypsin digestion followed by special stains such as G-banding, Q-banding and C-banding, as well as fluorescence staining whenever indicated.

Biopsy materials of skin, gonads, tumors are cultivated by tissue culture technique and cells harvested for chromosomal analysis.

Patient Material: The figures compiled below are for the last 12 months.

October 1977 - November 1978

Type of Material Examined:	Accessions	Smears
Vaginal and Cervical	942	1,575
Other (fluids, special procedures, special stains and chromosomal analysis)	2,869	7,697
Total	3,811	9,272

Sources of Material Received

Institutes	Accessions	Smears
NCI, NHLI, NIAID, NIAMDD, NIMH, NICHHD, NEI, NINCDS, EHS, NIDR and Referral Total	3,811	9,272

Major Findings: Major contributions of the Cytopathology Section:

1. Providing prompt services and early diagnosis in cytological materials.
2. Providing cytologic evaluation and estimated ranges of either relapse or remission in the continual management of meningeal leukemia.
3. Providing cytological evaluation of therapeutic effects on urinary specimens.
4. Providing chromosomal analysis for clinical studies as well as in the establishment of definitive malignancies.
5. Providing cytologic methods to research projects specifically, fine needle aspiration in thyroid nodules and bone lesions.

Significance to Biomedical Research and the Program of the Institute: 1. The diagnostic value of exfoliative cytology in the clinical management of various disease states is an established fact.

2. Aspiration cytologic techniques are useful in establishing diagnosis on metastatic diseases and/or recurrent malignancies. In such instances, the fine needle aspiration technique eliminates the more involved incisional biopsy and therefore is more economical to the clinicians as well as to the patients.

3. Chromosomal analysis is a way of establishing definitive diagnoses in selected patients with endocrine disorders, congenital defects and also in some malignancies.

Proposed Course: 1. Continue to provide a complete cytodiagnostic service for the entire staff of the Clinical Center in various research projects.

2. Continual improvement and development of established as well as new techniques in cytologic and cytogenetic fields.

Activities: From October 1, 1977 to November 1978

1. Lecture on Fine Needle Aspiration Cytology, November 8, 1977.

2. Seminar on Thyroid Aspiration Cytology, April 1, 1978.

3. Lecture and workshop in Fine Needle Aspiration of Breast Lesions; Tutorial in Clinical Cytology, Chicago, May 13-20, 1978.

4. Lecture on Exfoliative Cytology, June 5, 1978.

5. Lecture on Chemotherapeutic Effects expressed on cytology, June 6, 1978.

6. Present paper on "Studies of Cells in Fine Needle Aspiration of Thyroid Gland", November 7-11, 1978, American Society of Cytology, Miami, Florida.

Publications

1. Gershengorn, M.C., McClung, M.R., Chu, E.W., Hanson, T.A.S., Weintraub, B.D., and Robbins, J.: Fine needle aspiration cytology in the preoperative diagnosis of thyroid nodules. Ann. Intern. Med. 87: 265-269, 1977.

2. Tralka, T.S., Costa, J.C., Sindelar, W.F., Rabson, A.S., Gullino, P.M., Henson, E., Chu, E.W., and O'Conor, G.T.: Spontaneous lymphosarcoma arising in a nude mouse: Characterization in vivo and in vitro. J. Natl. Cancer Inst. 58: 977-982, 1977.

3. Chu, E.W., Gershengorn, M.C., McClung, M.R., Hanson, T.A.S., Brennan, M., Robbins, J., and Weintraub, B.D.: The role of fine needle aspiration cytology in the diagnosis of thyroid cysts and nodules. Radiation-Associated Thyroid Carcinoma 347-348, 1977.

4. Cheng, W.S., Cheng, A., Chu, E.W., and Peng, J.W.: The atypical fluorescent body of the interphase nuclei of buccal mucosal cells and its relationship to the Y chromosome. Acta Cytol. (in press)

5. Mills, S.R., Doppman, J.L., Head, G.L., Javadpour, N., Brennan, M.F., and Chu, E.W.: Transcatheter brush biopsy of intravenous tumor thrombi. Radiology (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00582-07 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Chronic Mouse Cytomegalovirus Infection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.E. Henson	Medical Director	LP NCI
OTHER:	R.E. Levinson	Biologist	LP NCI

COOPERATING UNITS (if any)

University of Cincinnati School of Medicine (Dr. R.D. Smith)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the possible reactivation of chronic mouse cytomegalovirus infection by immunosuppression and antilymphocytic serum (ALS). Administration of ALS shortly after acute infection leads to a chronic disseminated disease with glomerulonephritis.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Study the mechanisms of chronic murine cytomegalovirus infection in the salivary glands and lymphoid tissue of mice chronically infected with this virus and effect of anti-lymphocyte serum on infection.

Methods Employed: Tissue culture, immunofluorescence, electron microscopy.

Significance to Biomedical Research and the Program of the Institute: The mouse cytomegalovirus causes chronic infection in the submaxillary glands, lymph nodes, and spleens of many strains of mice. Infection in the lymph nodes and spleen probably occurs in lymphocytes. The murine cytomegalovirus belongs to the herpesvirus group of animal viruses, along with the Epstein-Barr virus and *Herpesvirus saimiri*.

Proposed Course: Part of this study is completed. Results indicate that chronic CMV infection does not enhance or accelerate natural development of lymphoma in mice. Present studies involve observations on the course of acute and chronic infection following the administration of anti-lymphocytic serum.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00591-04 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunofluorescence Studies on Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.E. Henson	Medical Director	LP NCI
OTHER:	R.E. Levinson	Biologist	LP NCI
	R.S. Adelstein	Chief, Molecular Cardiology Section	IR CB NHLBI
	M.A. Conti	Graduate Student	IR CB NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, Division of Intramural Research, NHLBI

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range of this project to study the differences of the myosins and other proteins present in normal tissue, rhabdomyosarcomas, osteogenic sarcomas, and other soft tissue tumors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Use of immunofluorescence on frozen section material to look for novel or unusual proteins synthesized by human tumors.

Methods Employed: Immunofluorescence immunology.

Major Findings: Dedifferentiated rhabdomyosarcoma cells produce a new type of myosin immunologically and biochemically related to myosin present in fibroblasts. Myosin might be helpful in diagnosis of rhabdomyosarcomas.

Significance to Biomedical Research and the Program of the Institute: The purpose is to search for novel proteins in tumors and to determine if these proteins can be useful in the early diagnosis of cancer, diagnosis of precancerous lesions, and for predicting prognosis.

Proposed Course: Test a variety of tumors by immunofluorescence using antibodies prepared against purified proteins that have been isolated from tumors, fetal tissue, normal organs, or from other sources.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00592-04 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

A Morphologic Investigation of Virus Induced Membrane Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Head, Ultrastructural Pathology Section (Surgeon)	LP NCI
OTHER:	J.C. Costa	Head, Surgical Pathology Section (Expert)	LP NCI
	A.S. Rabson	Director, DCBD, NCI (Pathologist)	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project attempts to elucidate the relationship between membrane antigens and an Fc receptor which appear in Herpes simplex type II-infected cultured human retinoblastoma cells. Further, both of these will be related to the increased Con A lectin binding ability of HSV II-transformed cells previously noted in the literature. The appearance of an Fc receptor and increased lectin binding following infection at a time when viral-directed membrane antigens are appearing suggests a relationship between these three membrane events. We suspect the Fc receptor is a viral-directed membrane-associated protein, as are the increased Con A binding sites. We are attempting to prove or disprove this assumption using ferritin and hemocyanin-labelled antibodies and lectins. The ability to bind host IgG may confer survival advantages to HSV-infected cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1. To characterize the membrane antigen (MA) of herpes simplex with regard to the time of appearance following infection, the distribution, and the association with lectin receptor sites, in cultured retinoblastoma cells.

2. To compare these results with those in the literature, and to determine the association, if any, of these membrane sites with the increased agglutinability of transformed cells by the lectin concanavalin A.

3. To compare the MA sites with the Fc receptor which also appears following HSV infection.

Methods Employed: High resolution scanning electron microscopy and transmission electron microscopy, utilizing ferritin or hemocyanin-tagged lectins or immunoglobulins.

Significance to Biomedical Research and the Program of the Institute: 1. The known increased agglutinability of transformed cells by plant lectins (such as concanavalin A) may be related to an alteration in number or distribution of cell surface receptors for lectins, which may coincide with the appearance of viral-associated glycoproteins in the membrane.

2. The HSV-directed Fc receptor may play a role in virus latency and oncogenesis.

3. A double label study, labelling both viral antigen-associated membrane sites and lectin binding sites, should provide unequivocal evidence, pro or con, of the means whereby viruses induce increased cell agglutinability.

Proposed Course: Considerable time will be spent preparing reagents for labelling the membranes and installing the high resolution SEM capability. Once the technical aspects are complete, the project will progress through labelling experiments (single and double labels) for viral membrane antigens, the viral Fc receptor, and Con A binding sites, which will be assayed by scanning and transmission electron microscopy. Quantity, distribution, and relationship one to another of each of the parameters will be compared.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00593-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunofluorescent Study of Cholera Toxin/Thyroid Stimulating Hormone Binding to Thyroid

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.E. Henson	Medical Director	LP NCI
OTHER:	R.E. Levinson	Biologist	LP NCI

COOPERATING UNITS (if any)

Case Western Reserve University School of Medicine (Dr. Brian R. Mullin)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to develop a probe for cell membrane-bound receptors whose presence is dependent on degree of differentiation in normal tissue and neoplasms. Cholera toxin and thyroid stimulating hormone are to be used to determine presence of receptors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Utilization of immunofluorescent techniques on human and bovine tissue in order to detect presence of membrane-bound receptors for cholera toxin and thyroid stimulating hormone in thyroid.

Methods Employed: Cholera toxin and thyroid stimulating hormone are allowed to bind to frozen thyroid sections under a variety of sets of conditions. Immunofluorescent labelling and microscopy are used to detect binding of CT/TSH.

Major Findings: Cholera toxin will block the binding of TSH to thyroid tissue or compete successfully depending on conditions.

Significance to Biomedical Research and the Program of the Institute: Cholera toxin receptors have been shown to disappear from membranes of cells in culture at stages of differentiation. Transformed and tumor cells have a different receptor composition from normal cells. The cholera toxin probe can be used as a marker for differentiation and diagnosis in a variety of tumors. This follows the investigation of soft tissue sarcomas this laboratory is presently engaged in. The CT/TSH competition provides information as to the specific nature of the receptors detected by this technique.

Proposed Course: Compare nature of binding of cholera toxin to normal tissue versus that of tumor tissue.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00594-02 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Pathology of Family Cancer Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.E. Henson	Medical Director	LP NCI
OTHER:	D. Parry	Visiting Scientist	CEB NCI
	J.J. Mulvihill	Head, Clinical Genetics Section	CEB NCI
	J.F. Fraumeni, Jr.	Chief, Environmental Epidemiology Branch	EEB NCI
	W.A. Blattner	Clinical Investigator	EEB NCI

COOPERATING UNITS (if any)

Epidemiology Branch, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 5	PROFESSIONAL: 5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Review histopathology material from families with high incidence of cancer. These families are being followed by the Epidemiology Branch.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Review and confirm the histological diagnoses on families that have a high incidence of cancer and which are being followed by the Epidemiology Branch.

Methods Employed: Light microscopy, special histochemistry stains.

Significance to Biomedical Research and the Program of the Institute: Confirm diagnoses of cancer and apply uniform terminology and classification to the lesions so that patterns may be found.

Proposed Course: Continue to review surgical pathology or autopsy material submitted from referring hospitals and report results to the Epidemiology Branch.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00595-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Liposarcoma, a Study of the Ultrastructure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.L. Reddick	Surgeon	LP NCI
OTHER:	H.J. Michelitch	Biologist	LP NCI
	T.J. Triche	Head, Ultrastructural Pathology Section (Surgeon)	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study is to provide the 1st comprehensive study of the ultrastructure of the various types of liposarcoma (round cell, myxoid, pleomorphic, sclerosing type)

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To define the ultrastructural characteristics of the various subtypes of liposarcoma.

Methods Employed: Electron microscopy.

Major Findings: Are similar characteristics but subtypes differ in lipid filled cells and background matrix.

Significance to Biomedical Research and the Program of the Institute: Aid in differential diagnoses from other myxoid tumors.

Proposed Course: To gather additional materials on these cases.

Publications: In preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00596-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Osteosarcoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.L. Reddick	Surgeon	LP NCI
OTHER:	H.J. Michelitch	Biologist	LP NCI
	A.M. Levine	Clinical Associate	SURG NCI
	T.J. Triche	Head, Ultrastructural Pathology Section (Surgeon)	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Study of the ultrastructural characteristics of osteogenic sarcoma to evaluate the tumors both of the primary tumor and the metastatic tumor.

Project Description

Objectives: To evaluate the ultrastructural characteristics of the various subtypes of osteogenic sarcoma.

Methods Employed: Electron microscopy and tissue culture.

Major Findings: Are differences in the basic makeup of the various subtypes of osteogenic sarcoma in contrast to published observations that all tumors are basically the same.

Significance to Biomedical Research and the Program of the Institute: First attempt to adequately subclassify osteogenic sarcomas by EM as they relate to the light microscopic findings.

Proposed Course: Continue study to get additional samples of some of the lesser subtypes to include as part of study.

Publications: In preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00597-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Malignant Soft Tissue Sarcomas: Ultrastructural Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.L. Reddick	Surgeon	LP NCI
OTHER:	T.J. Triche	Head, Ultrastructural Pathology Section (Surgeon)	LP NCI
	H.J. Michelitch	Biologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To define the similarities and differences to aid in diagnosis of pleomorphic soft tissue sarcomas (malignant fibrous histiocytoma, pleomorphic rhabdomyosarcoma, pleomorphic liposarcoma)

Project Description

Objectives: To define the similarities and differences to aid in diagnosis of pleomorphic soft tissue sarcomas (malignant fibrous histiocytoma, pleomorphic rhabdomyosarcoma, pleomorphic liposarcoma).

Methods Employed: Electron microscopic examination, and tissue culture studies.

Major Findings: Pleomorphic sarcomas have a similar cellular composition but do differ ultrastructurally such that a diagnosis can be made even on those extremely pleomorphic tumors.

Significance to Biomedical Research and the Program of the Institute: Aids in diagnosis, classification of tumors and may aid in proper selection of therapeutic modalities for protocol patients.

Proposed Course: Continue studies as patients come to NIH for protocol studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00598-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

A Retrospective Study of Non-Hodgkin's Lymphomas Treated at the NIH

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.J. Garvin	Surgeon	LP NCI
OTHER:	C.W. Berard	Head, Hematopathology Section - Medical Director	LP NCI
	R. Jones	M.D.	M NCI
	R. Young	M.D.	M NCI
	R. Simon	M.D.	DCT NCI
	J. Merrill	M.D. (Previous Clin. Assoc.)	M NCI
	K. Osbourne	M.D. (Previous Clin. Assoc.)	M NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to review all of the clinical and pathologic data of all patients treated with non-Hodgkin's lymphoma at the NIH since the opening of the Clinical Center in order to: 1) characterize the population; 2) understand the natural course of the disease, e.g. histologic progression; 3) evaluate current histologic classifications; and 4) correlate treatment and staging data with survival.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Clinical-pathological study of patients with non-Hodgkin's lymphomas in relationship to their natural course, current classifications and clinical treatment.

Methods Employed: The histologic slides and clinical charts are all reviewed to classify the tumors and obtain pertinent clinical information. The data is coded and evaluated by computer analysis.

Major Findings: There is a histologic progression of a portion of non-Hodgkin's lymphomas over a period of time to a histologic type with a poorer prognosis.

Significance to Biomedical Research and the Program of the Institute: The research will help establish the natural clinical course of non-Hodgkin's lymphomas. The efficacy of the current histologic classifications for non-Hodgkin's lymphomas will be tested on the clinical course.

Proposed Course: The project is still in effect. Data is currently being analyzed by computer.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00599-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Isolation, Purification, and Identification of the HY Antigen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.J. Garvin	Surgeon	LP NCI
OTHER:	R. Camerini-Otero	M.D.	A&R NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The HY antigen is believed to be the significant factor in normal sexual differentiation of the gonads. To better understand the mechanisms of differentiation, attempts are being made to purify the antigen from mice, define it biochemically and localize it by electron microscopy.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To purify and localize the HY antigen in mice.

Methods Employed: Antisera is made by injecting inbred strains of female mice with male spleen cells. The antisera will be used to establish an assay for the biochemical purification.

Major Findings: Antisera is currently being produced in mice.

Significance to Biomedical Research and the Program of the Institute: An understanding of the HY antigen will lead to understanding of normal differentiation mechanisms and aberrant development which leads to dysgenesis or neoplasms.

Proposed Course: The project is still in effect. Antisera production and the establishment of an assay are the immediate goals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00600-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Secretion and Uptake of Lysosomal Enzymes by Human Diploid Fibroblasts in Tissue Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.J. Garvin	Surgeon	LP NCI
OTHER:	E.F. Neufeld	Head, Section of Human Biochemical Genetics	A&R NIAMDD
	L.H. Rome	Staff Associate, Section of Human Biochemical Genetics	A&R NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to identify the mechanism by which lysosomal enzymes are secreted and taken up by normal human fibroblasts in tissue culture. Cell fractionation and ultrastructural cytochemistry have been used to identify lysosomal enzymes and study their production. GERL has been identified in normal fibroblasts and its separation by centrifugation has been established.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To identify the mechanism by which normal human fibroblasts secrete and adsorb lysosomal enzymes.

Major Findings: Two populations of lysosomes can be identified by biochemical and ultrastructural cytochemical methods of cell fractions. In addition, GERL has been identified in human tissues for the first time by cytochemical study of intact cells.

Significance to Biomedical Research and the Program of the Institute: The identification of two populations of lysosomes (one being GERL) in normal fibroblasts is a significant advancement in understanding the function of the GERL, Golgi apparatus and lysosomes. An understanding of these mechanisms will lead to the pathogenesis of some genetic diseases as well as rheumatoid arthritis.

Proposed Course: The project is still in progress. Further markers are being explored to understand these mechanisms.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00601-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Determination of Oligosaccharides in Urine of Pregnant and Lactating Women by Radioimmunoassay

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Expert	LP NCI
OTHER:	V. Ginsburg	Chief, Section on Biochemistry	LBP NIAMDD

COOPERATING UNITS (if any)

Department of Clinical Chemistry, University of Lund, Lund, Sweden
(Dr. Arne Lundblad)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a recent report (Hallgren et al., J. Biol. Chem. 252: 1014-1022, 1023-1033, 1034-1040, 1977) described the isolation and chemical structure of several oligosaccharides in urine from 3 pregnant or lactating women. Some of these were oligosaccharides with human Le^a or Le^b blood group activity. Using a radioimmunoassay developed in our laboratory we examined the urinary excretion of Le^b-active oligosaccharides in urine of 138 pregnant or lactating women of different Lewis blood types. The amount of Le^b-active oligosaccharides in urine during pregnancy and lactation correlates with Lewis blood type and with the strength of expression of Le^b antigen on erythrocytes. The site of origin of these oligosaccharides has not been defined but several lines of indirect evidence suggest they may originate in mammary gland.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To determine qualitative and quantitative patterns of urinary excretion of Le^b-active oligosaccharides during pregnancy and lactation.

Methods Employed: A radioimmunoassay method using goat antiserum prepared by immunization with a synthetic glycoconjugate containing the Le^b-active oligosaccharide lacto-N-difucohexaose I as previously described (Zopf, D.A. et al., J. Immunol. 115: 1525, 1975).

Major Findings: 1) Radioimmunoassay can be used as a convenient method for accurate determination of oligosaccharides in unpurified urine. 2) Excretion of Le^b-active oligosaccharides increases gradually during pregnancy, reaches a maximum in the third trimester and early postpartum period, and continues until lactation ceases. 3) Amounts of Le^b-active oligosaccharides in urine correlates with Lewis blood group and with the strength of expression of Le^b antigen on red blood cells.

Significance to Biomedical Research and the Program of the Institute: This is the first demonstration of the utility of the radioimmunoassay method for detecting specific oligosaccharides in a complex mixture. It is also the first quantitative description of excretion of urinary oligosaccharides and their correlation with blood type. The available evidence suggests that mammary gland may be the ultimate source of these oligosaccharides, and that they might provide an index of metabolic activity of breast epithelial cells.

Proposed Course: The project is still in effect. Additional studies will be carried out using radioimmunoassays for an oligosaccharide whose excretion is probably not related to blood type, lacto-N-tetraose.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00602-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immune Response of CBA/N Mice to Isomaltosyl Oligosaccharides Coupled to Protein Carriers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP NCI
	K. Stein	Staff Fellow	LI NIAID
OTHER:	W.E. Paul	Chief	LI NIAID

COOPERATING UNITS (if any)

Laboratory of Immunology, NIAID

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

CBA/N mice are an inbred strain of animals that exhibit an X-linked deficiency in immune responsiveness to certain carbohydrate antigens including dextrans. Isomaltodextrins derived by partial enzymatic or acid hydrolysis of dextran were coupled as haptens to the protein carrier keyhole-limpet hemocyanin and were used as immunogens. These glycoconjugates were used to study formation of antibodies that bind dextran in normal BALB/c and (CBA/N X BALB/c) F_1 females and males.

Project Description

Objectives: To investigate immune responsiveness of CBA/N and normal mice to isomaltodextrin oligosaccharide determinants coupled to a protein carrier.

Methods Employed: Isomaltodextrins are prepared by partial enzymatic or acid hydrolysis of *Leuconostoc mensenteroides* strain 512. Oligosaccharides are derivatized with β-(p-aminophenyl) ethylamine and coupled to KLH after activation to the isothiocyanate. Immune responses are monitored by isoelectric focusing of mouse sera or hemolytic plaque assays of spleen cells.

Major Findings: Antibodies that bind dextran can be elicited by immunization with isomaltotriosyl-KLH in CBA/NX Balb F_1 males and females as well as in normal Balb mice.

Significance to Biomedical Research and the Program of the Institute: The immune deficiency of CBA/N mice for response to polysaccharide antigens apparently can be circumvented when polysaccharide determinants are presented via a "T cell dependent" route. This finding opens a possible route to vaccination against bacterial organisms (e.g. pneumococcus) with surface polysaccharides that contain abundant repeating carbohydrate sequences but which may be poorly immunogenic in their native state.

Proposed Course: The effects of length of oligosaccharide chain, epitope density, and modification of protein carrier will be investigated. Documentation of an apparent difference in IgG subclasses between responding and CBA/N X Balb F_1 females and males will also be carried out.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00603-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Specific Covalent Fluorescence Labelling of Cell Surface Galactose Residues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP NCI
OTHER:	W.W. Stewart	Staff Fellow	LEP NIAMDD
	J.C. Costa	Expert	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Vero cells grown in culture are treated with galactose oxidase to generate aldehyde moieties in the 6-position of non-reducing terminal galactose and galactosamine residues in surface glycoproteins and glycolipids. Subsequent treatment with Lucifer yellow carbohydrazide and reduction with sodium borohydride confers a fluorescence label to glycoconjugates in the cell surface membrane.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop a method for fluorescence labelling of surface glycoconjugates in living cells in order to follow their migration by direct observation and by fluorometry of purified cell fractions.

Methods Employed: Commercial preparations of galactose oxidase were used on Vero cells maintained in our laboratories in continuous culture. Lucifer yellow carbohydrazide was prepared by Walter Stewart.

Major Findings: Bright surface fluorescence is seen in cells treated with galactose oxidase than with Lucifer yellow carbohydrazide, a fluorescence dye with high fluorescence yield. No label is seen if galactose oxidase is omitted or if enzyme treated cells are treated with sodium borohydride before Lucifer yellow carbohydrazide. Labelled cells remain viable for several days.

Significance to Biomedical Research and the Program of the Institute: This method offers a way to examine the fate of cell surface glycoproteins in living cells at intervals after labelling. Specifically it will enable us to examine the question: Do viral glycoproteins appear as host cell surface components prior to incorporation into the virus particle?

Proposed Course: This project was begun recently. Documentation of the chemical basis for labelling and fluorometric assay of cell fractions are in progress.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00504-22 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

An Electron Optical Investigation of Normal and Pathologic Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.G. Banfield	Medical Officer - Pathology	LP NCI
OTHER:	J. Peng	M.D.	MO NCI
	C.J. Dawe	Head, Comparative Oncology Section (Med. Officer (Path.))	LP NCI
	C.W. Lee	Biologist	LP NCI
	E. Lee	Biologist	MO NCI
	T.S. Tralka	Biologist	LP NCI
	A.S. Rabson	Director, DCBD - Pathologist	LP NCI
	J.D. Small	D.V.M.	VR R
	J.C. Sutherland	M.D.	BCRC NCI
	T. Kuwabara	M.D.	LVR NEI

COOPERATING UNITS (if any)

Cytogenetic Oncology Section, Medicine Branch (Dr. Peng)
Division of Research Services, Veterinary Resources Branch (Dr. Small)
Laboratory of Vision Research, National Eye Institute (Dr. Kuwabara)

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8	4	4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The transmission electron microscope is applied to problems directly or peripherally related to neoplasia. A major effort has been a study of the lamella-particle complex which has been found in human and animal lymphoma cells and human term placenta. It has also been recognized in tissue culture cells infected with Herpesvirus saimiri and as an apparently normal component of the horizontal cells of the human and beagle retina.

Characterization of a hematopoietic stem cell in human peripheral blood is being attempted.

The electron microscopy of a snake chondrosarcoma has been completed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1. To describe and interpret the ultrastructure of selected pathologic states and to compare the results with suitable controls.

2. To look for and study the inciting agent or accompanying agent in neoplastic and other disease states using human, animal and tissue culture material.

Major Findings: A complex consisting of ribosome-like particles and a striated lamella occurring in stacked, tubular, and twisted ribbon-like configurations was described in the nuclei of confluent monolayer cultures of primary owl monkey kidney cells infected with Herpesvirus saimiri. Attention was also directed to a similar structure normally present in the horizontal cells of human and beagle retinas, Kolmer's crystalloids, and that all these structures were similar to those found in the lymphoma cells of the northern pike.

There is another lamella-particle complex found in human lymphoma cells but the lamellae are different from those in Herpesvirus, retina and northern pike lymphoma in that they are thinner, there is no clear-cut periodicity, there are regularly spaced indistinct tubular structures longitudinally in the lamellae and the ribosome-like component is more variable. However, a cylindrical structure like those found in human leukemic cells was found in mesenchymal cells closely associated with the vessels in 2 of 10 human term placentas.

Significance to Biomedical Research and the Program of the Institute: The particles in the lamella-particle complex have been shown to be ribosomes in Kolmer's crystalloids and in hairy cell leukemia. It is likely that the particles are ribosomes in all the lamella-particle complexes so far described with the exception of Herpesvirus saimiri, a DNA virus. This would suggest that the complex is a site of synthesis of some cellular product. The relation of this structure to the leukemic process or cell function may be of importance.

In collaboration with Dr. Peng, the attempt to isolate and characterize by electron microscopy a hematopoietic stem cell in human peripheral blood has not been successful. Positive structural identification of such a cell would open up new lines of investigation especially as applied to neoplasia.

In collaboration with Drs. Small and Dawe the cells of a corn snake chondrosarcoma contained spherical and tubular clusters of 400 Å particles which persisted through two cultures but were not found thereafter. This is an original observation of a new structure the nature of which is unknown.

Proposed Course: 1. Attempt isolation of hematopoietic stem cells from a larger volume of peripheral blood.

2. Pursue the lamella-particle complex investigation as circumstances dictate.

3. Complete the snake chondrosarcoma manuscript.

Publications

Banfield, W.G., Lee, C.W., Tralka, T.S., and Rabson, A.S.: Lamella-particle complexes in nuclei of owl monkey kidney cells infected with Herpesvirus saimiri. J. Natl. Cancer Inst. 58: 1421-1425, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00505-08 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Pulmonary Carcinogenesis: Testing and Examining the Mechanisms Through Animal Models

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT*

PI:	M.F. Stanton	Medical Officer - Research	LP NCI
OTHER:	E. Miller	Biologist	LP NCI
	M. May	Biologist	LP NCI

COOPERATING UNITS (if any) Johns-Manville Res. & Engineering Center, Manville, N.J.; Owens-Corning Fiberglass Corp., Toledo, Ohio; Roswell Park Mem. Center for Cancer Research; Pharmacopathics Inc.; Richard B. Russell Agricult. Res. Center, USDA, Athens, Ga., Natl. Environ. Res. Center, EPA, North Carolina

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This program is designed to investigate the major causes and mechanisms of pulmonary cancer in man through the study of lung cancer induced in animals. A variety of inhaled air-pollutants that are petroleum-soluble are being studied by incorporation into melted beeswax which can be infiltrated into the lung or other organs to be retained with slow release over long periods of time. Secondly, we continue to investigate those properties of fibers critical to pulmonary and pleural carcinogenesis. Factors of special concern this year have been the role that macrophages play in particulate invasion of the lung.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Investigations into pulmonary carcinogenesis with the suspect pulmonary carcinogens for man and the factors that might influence their effect.

I. Wax Infiltrates of the Lung

In the studies with intrapulmonary deposits of cigarette smoke condensates, a large series involving more than 4,000 rats has been completed to determine the carcinogenic potential of 9 highly defined fractions of the PAH of cigarette smoke condensate isolated by the Agriculture Research Center, USDA, Athens, Georgia.

Tobacco Carcinogenesis: Results of our tests with cigarette smoke condensate confirm that while the PAH fraction is by far the most carcinogenic component, other components, particularly the acidic fraction and aqueous methanol subfraction, augment carcinogenesis and are in themselves capable of pulmonary tumor induction. However, the fact that they augment pulmonary carcinogenesis would suggest that the removal of any of several components of either the acidic or neutral fractions of CSC might reduce carcinogenicity significantly. The results are also of significance in supporting the similar findings of skin painting experiments, thus reinforcing the idea that skin painting experiments are a valid means of determining pulmonary carcinogenesis.

Pulmonary Carcinogenesis with Air Pollutants: Similar studies to those above with extracts of collected air particulates have been surprisingly successful. The PAH crude extract obtained from the EPA proved to be highly carcinogenic for the lung. We are now in the process of testing the refined fractions from this crude condensate. However, an overly ambitious attempt to alter the pellet composition by adding DMSO may prove disappointing. There is no doubt that the composition of the vehicle is critical to this technique.

Other Explorations: For some time now it has been challenging to learn: 1) How small a deposit of carcinogen impregnated wax would be carcinogenic; and 2) What would be the response of other organs. These two projects are now underway. Hopefully they may be instrumental in the development of model cancers in such organs as the pancreas and salivary glands.

II. Fiber Carcinogenesis

Despite the handicap of working in temporary facilities pending remodeling and the uncertainty of animal housing that have continued through the year, progress has been made in several of the areas outlined last year.

As previously reported, it is our thesis that the essence of fiber carcinogenicity lies in the durability and dimension of the fiber. Current correlation studies would indicate that maximum carcinogenicity resides in fibers < 0.25 μm dia. x > 8 μm length. However, to be of practical significance these figures need to be greatly refined. We have recently examined and tested several ceramic fibers that have the great advantage of being of remarkably uniform diameter. We have not been able to obtain exceptionally fine fibers of this type although they could be synthesized. At diameters of 3 μm, the fibers are not carcinogenic. It seems invaluable at this point to obtain uniform ultrafine ceramic fibers. There also is evidence of a minimal size limit to fiber carcinogenicity, i.e. fibers less than 0.02 μm in diameter tend to ball-up and cease acting as a fiber--consequently we have seen only minimal evidence of cancer from attapulgite and halloysite.

The project with intrapleural applications of asbestos and fibrous glass to BALB/c mice has been successfully completed with the establishment of another species carcinogenic to both fibrous glass and asbestos. Lack of carcinogenesis with the large diameter glass further confirms that this phenomenon is not merely an example of "foreign body" carcinogenesis.

Finally, a major effort has been into refining methods for the determination of fiber dimension and size. After some exploration, we have settled on a system of two montages at approximately 100x and 500x on the same fields--permitting us to insure that all sized fibers are included in the count. Errors and changes in the calibration of the scope from hour to hour has been a sizeable problem. Our most recent approach has been used on 13 crocodolite samples that have a wide range in carcinogenicity. All that remains to do now is the counting and calculating. Perhaps this will be reserved for when the lab becomes completely active.

Publications

Stanton, M.F., Layard, M.W., Miller, E., May, M., and Kent, B.: The carcinogenicity of fibrous minerals. National Bureau of Standards Special Publication. Proceedings of the *Workshop on Asbestos: Definitions and Measurement Methods*, held at the National Bureau of Standards, Gaithersburg, Maryland, July 18-20, 1977. Issued January, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00516-13 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Comparative Studies on Neoplasms in Relation to Pathogenesis, Etiology and Diagnosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.F. Stanton	Medical Officer - Research	LP NCI
OTHER:	R.H. Adamson	Chief	LCHPH NCI
	E. Miller	Biologist	LP NCI
	M. May	Biologist	LP NCI
	C.M. Poore	Biologist	LP FCRC
	B. Coolidge & Staff	Technologists	LP NCI, FCRC

COOPERATING UNITS (if any)

LCHPH, NCI
Pathological Technology Section, LP, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a general program in the diagnosis and recognition of behavioral patterns in the tumors of laboratory animals employing techniques in light and electron microscopy and histochemistry. Major interest is currently focused on tumors of the Osborne-Mendel rat, since more than 4,000 aged females of this strain have been necropsied in this laboratory.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: A study and accessioning of cancer in laboratory animals. Collection and development of competence in special areas of cancer morphology. Studies of histopathological and fine structure alterations related to the pathogenesis of disease induced by oncogenic viruses and other carcinogens.

Major Findings: This year a new project was initiated primarily to understand several common but poorly studied tumors of the O-M rat. At the terminal sacrifice of non-inbreds on other experiments any rats with unique tumors had their tumors removed and fixed for EM and additionally grafted into our collection of inbred O-Ms. To date we have had no success at grafting 6 tumors; however, we feel that our chances of success are moderately good. Graft failure does not prevent EM analysis of course. Our major interest is in the lipo-reticular sarcoma that is relatively common in the kidney of this strain. All varieties of lung tumors are under study as well. The goal of finding a simple method for grafting and maintaining tumors of non-inbred animals is being investigated.

An ongoing program has been the development of special competence in the histopathological and fine structural aspects of tumors of the lung, bone and mammary glands of rodents. Collection and examination of induced and naturally occurring bone tumors has been in progress for more than 5 years and will continue.

Proposed Course: Tumors of all types will be transplanted into inbred rats in an effort to learn more about specific tumors. We have through the past 5-6 years acquired an extensive catalog of neoplasms of all types that develop in aged female Osborne-Mendel rats. This data will be collated in an effort to establish normal incidence rates for our colony. In addition, time will be devoted to training in electron microscopy, studies of neoplasia in lower animals and preparation of a monograph on lung tumors in rats.

Publications

1. Stanton, M.F.: Tumors of the lung. WHO Monograph Pathology of Tumours in Experimental Animals, vol. I, pt. II, Tumours of the Rat. 1976. International Agency for Research on Cancer, Publication #6.

2. Stanton, M.F.: Tumors of bone. WHO Monograph Pathology of Tumours in Experimental Animals, vol. II, Tumours of the Mouse. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00521-08 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Epithelial-Mesenchymal Interactions in Neoplastic Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.J. Dawe	Head, Comparative Oncology Section - Med. Officer (Path.)	LP NCI
OTHER:	J.J. Peng	Head, Cytogenetic Oncology Section	M NCI
	S. Fisher	Post-doctoral Fellow	LP NCI
	W.D. Morgan	Biologist	LP NCI
	J.E. Williams	Biologist	LP NCI
	C.M. Poore	Biologist	LP NCI
	T. Sakakura	Former Visiting Fellow (now at Aichi Inst. for Cancer Research, Nagoya, Japan)	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 1/2	2 1/2	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is an investigation of the effects of interactions between epithelium and stroma during transformation of epithelium from normal to neoplastic state. The systems studied are: 1) Tumors of the submandibular salivary gland of mice, induced by polyoma virus; 2) Mammary tumors of mice, spontaneous or induced by mammary tumor virus (MTV). The methods used involve neoplastic transformation of epithelium in the presence or absence of specific types of mesenchyme (stroma). The results are evaluated on the basis of morphological and biological properties of the resulting neoplasms. Findings are that neoplastic changes induced in salivary epithelium by polyoma virus have widely variable characteristics, depending on whether the transformational and post-transformational events take place in the presence or absence of stromal cells. Stromal cells are not necessary for epithelial cell transformation, but epithelium transformed in a microenvironment devoid of other cellular components gives rise to tumors that are morphologically more atypical and biologically more aggressive than tumors induced either in vivo or in organotypic culture. The evidence suggests that stromal components may act to inhibit or decelerate tumor progression and to influence the type and degree of differentiation expressed by the neoplastic cells

Project Description

Objectives: Most neoplasms, like normal tissues, show the ability of their stem cells to undergo some degree of differentiation and to manifest some amount of functional activity. Developmental biologists have shown that an important determinant in normal morphogenesis and differentiation of glandular organs is the interaction between the epithelial and mesenchymal components. Therefore it should follow that the nature of epithelial-mesenchymal interaction in progress when neoplastic transformation of epithelium takes place will in some degree affect the kind and the extent of differentiation of the resulting tumor. The object of this project is to test this hypothesis and to determine whether interference with epithelio-mesenchymal interactions can modify the effectiveness of carcinogenic agents.

Methods Employed: Work with the two systems listed above is separately described below, for each.

A. Work primarily originated in this laboratory: Polyoma virus/mouse salivary gland system.

1. Methods for preparing polyoma virus, inducing tumors in vivo, and transforming salivary epithelium in vitro have been described in previous Annual Reports and in published papers (See Dawe, C.J.: Epithelial-mesenchymal interactions in relation to the genesis of polyoma-induced tumours of mouse salivary gland. In Tissue Interactions in Carcinogenesis, D. Tarin, (ed.), Academic Press, 1972, pp. 305-353. Tumors induced in vivo are compared morphologically with those derived from salivary epithelium transformed in vitro, by light and transmission electron microscopy. Tumors of these two different derivations are compared biologically in the following ways:

(a) In necropsy studies which determine whether the tumors are locally invasive and/or produce distant metastases.

(b) In studies of the interaction of tumor cells with normal embryonic salivary epithelium. These are done by placing isolated salivary epithelial rudiments within solid tumor explants and transplanting the combination subcutaneously to syngeneic newborn mice. After 14 days in the new host, the combinations are recovered, fixed, serially sectioned, and evaluated for evidence of morphogenesis and/or cytodifferentiation in the embryonic epithelium. In an earlier study (Dawe et al., in Epithelial-Mesenchymal Interactions; R. Fleischmajer, (ed.), Williams & Wilkins Co., 1968), it was found that salivary tumors induced by PV in vivo supported morphogenesis and some cytodifferentiation in normal salivary epithelium. In the present work, this experiment was repeated, but using tumors resulting from transformation of salivary epithelium in vitro.

(c) In studies of the interaction of tumor cells with normal embryonic salivary, dental, and skin mesenchymes, respectively. These studies are performed as in (b) above, but substituting mesenchyme for epithelium. Previous work (see ref. in (b)) had shown no effect of normal mesenchyme on salivary tumors induced *in vivo*.

(d) An additional set of controls has been added to the study. These consist of lines of salivary tumors induced by PV *in vivo*, then subsequently subjected to the same conditions *in vitro* which pertained to the experiments in which isolated epithelium was transformed *in vitro*. These tumor lines (transformed *in vivo*) are grown on Millipore filters for 30-60 days, then on glass for periods of 6 months and more. The resulting culture cell lines are then transferred back to syngeneic mice to determine how their morphological and biological properties compare with those of tumors derived from salivary epithelium transformed in isolation on Millipore filters and subsequently grown on glass.

(e) Dr. Fisher is preparing to compare isozyme systems in the tumors induced *in vivo* with those in tumors resulting from transformations *in vitro*. These studies will be described in next year's report.

Major Findings:

(a) Tumors derived from salivary epithelium transformed by PV on Millipore filters *in vitro* were found to differ sharply from salivary tumors induced by PV *in vivo*. Four lines transformed *in vitro* in the absence of mesenchyme gave rise to totally undifferentiated neoplasms. Of these epithelial lines transformed in the presence of mesenchyme on the opposite side of the membrane, one was undifferentiated, while one yielded tumors that were a mixture of undifferentiated neoplasm, squamous cell carcinoma, and adenocarcinoma. The third yielded tumors that, in early passages were mixtures of undifferentiated neoplasm, adenocarcinoma, and bone and cartilage-forming tumor cells. All 7 tumor lines contained large proportions of aneuploid cells, while tumors induced *in vivo* were composed of diploid and near-diploid cells.

Biologically, tumors derived from all 7 lines transformed *in vitro* were invasive in adjacent muscle and skin, whereas none of hundreds of tumors induced *in vivo* have been seen to invade these or other tissues. Tumors from the 7 lines transformed *in vitro* were frequently seen to metastasize to lungs and liver, and occasionally to lymph nodes. Only extremely rarely have salivary gland tumors induced *in vivo* by PV been found to metastasize, and then only to lung. It is concluded that the epithelial cells transformed by PV *in vitro* are both morphologically and biologically different from tumors induced in similar cells *in vivo*.

(b) Tumors derived from a line of salivary epithelium transformed *in vitro* in the absence of mesenchyme were found to support only a minimal degree of morphogenesis in normal salivary epithelium with which it was interacted. This contrasted distinctly with the extensive degree of morphogenesis (branching, duct and gland formation) seen in normal salivary epithelium interacted with tumors induced *in vivo*.

(c) Salivary, dental, and skin mesenchymes all failed to induce morphogenetic or cytodifferentiative changes in tumors derived from salivary epithelium transformed in vitro in the absence of mesenchyme. In this respect, the lines transformed in vitro did not differ from those transformed in vivo.

(d) In control experiments in which tumor induced in vivo was grown in culture conditions similar to those in which in vitro transformation was induced, the tumors derived from cultures took on properties closely resembling those of tumors derived from isolated epithelium transformed in vitro. This experiment has been done twice with a single tumor at different passage levels, with the same results in both trials. The tumors derived in this way were anaplastic, invasive, and showed squamous as well as undifferentiated components. Hence it was possible to develop tumors of comparable type from either of the following sequences:

(a) Salivary epithelium + Polyoma virus In vitro ⟶ Transformed cells ⟶ Growth on Millipore ⟶ Growth on glass ⟶ (on transfer of culture cells to newborn mouse) Undifferentiated and squamous cell carcinoma (mixed), highly aneuploid, invasive.

or (b) Salivary epithelium (in intact newborn mouse) + Polyoma virus In vivo ⟶ Salivary tumor (non-invasive, near diploid) ⟶ Cell culture line on Millipore ⟶ Cell culture line on glass ⟶ (on transfer to NB's) Undifferentiated and squamous cell carcinoma (mixed), highly aneuploid, invasive.

Significance to Biomedical Research and the Program of the Institute: The results show that:

(a) The same oncogenic virus acting on the same target cell (salivary epithelium) can induce morphologically and biologically different neoplasms if microenvironmental conditions are varied during and/or after the transformational events.

(b) Morphologically and biologically similar neoplasms can be obtained whether the microenvironmental variables are applied during and after transformation, or only after transformation. This suggests but does not prove that the primary transformational events are the same, regardless of microenvironmental variables, but that microenvironmental factors are important as modifying influences which act to cause what may be termed confluent tumor progression. I.e., the same end result can be reached via different routes with respect to the time matrix, with all factors such as oncogenic agent, target cell, microenvironmental factors, and tumor hosts being the same, but "juggled" in time sequencing.

An especially interesting aspect of the results is the fact that on the one hand we were unable to intentionally induce either morphogenesis or cytodifferentiation by interacting tumor with a "natural" inducer such as salivary, dental, or skin mesenchyme. On the other hand, simply by placing tumor cells in culture, first on millipore membrane, then on glass, it was possible to cause the tumors to differentiate toward squamous epithelium even though tumors induced in vivo and serially transplanted in vivo have never done this. Some factor(s) in the cell culture environment must therefore be influential in directing differentiation and altering the biological properties of tumor cells. An interesting recent observation relating to this point is that one of our cell lines transformed in vitro has apparently undergone such extensive differentiative change that it now keratinizes in vitro, as evidenced by Papanicolau stains, and concomitantly has slowed in growth rate to the point where it is now difficult to accomplish subcultures and maintain the line. In other words, the cell line appears to be committing suicide by undergoing excessive squamous differentiation.

It would obviously be useful to find the mechanism of this phenomenon so that tumors in vivo could be induced to undergo self-extinction or at least self-retardation through differentiation. Curiously, as in our own experience, efforts by others to induce differentiation in neoplastic cells by applying "natural" differentiative factors have failed, while "unnatural" factors such as culture environment alone, dimethylsulfoxide, hexamethyl bisacetamide, butyrate, mitomycin C, BrDU, and many others have inexplicably caused differentiation in certain neoplastic cells such as neuroblastomas, melanomas, and undifferentiated hematopoietic (reticulum cell) neoplasms.

B. Other work, collaborative in nature, related to this project.

(1) Dr. Jules Berman and collaborators in DCCP have been studying collagen production in continuous culture cell lines of liver cells and of hepatomas, in the presence and absence of added ascorbic acid. They found that cultured liver cells produce collagen fibers demonstrable with silver stains and transmission electron microscopy. Connective tissue cell lines also produced collagen under similar conditions, but in different patterns in relation to the cells than was seen for liver cells or liver tumor cells. As controls we contributed several of our epithelial and mesenchymal cell lines derived from transformation in vitro by polyoma virus.

It was found that PV-transformed salivary epithelium also produced collagen fibers in the presence of ascorbic acid, in much the same pattern as did liver cells. Dr. Berman is extending his study to determine which of the 4 main types of collagen is produced by each of the various cell lines.

A report on the findings concerned with production of collagen fibers, type not determined, is in press (Berman et al.).

(2) Dr. Teruo Sakakura, in continuation of studies started here, has completed a project in which it was shown that the adult mammary fat pad in mice is able to support development of embryonic mammary epithelium into virtually normal mammary gland, which secretes milk after pregnancy in the

host. This work indicates that even in old adult females the mammary mesenchyme retains properties of embryonic mammary mesenchyme. A report of this work has been submitted for publication (see publications list).

(3) The project undertaken by Dr. Michael Orlando, concerned with the histo- and cytogenesis of mammary tumors induced in nude mice infected at adult ages with polyoma virus, has not progressed significantly in the past year because of Dr. Orlando's time limitations while completing his residency in Clinical Pathology. However, relatively little remains to be done to complete the study. Dr. Orlando has now fixed small, early lesions of the mammary duct system in glutaraldehyde for electron microscopy. After these are examined and interpreted in correlation with the light microscopic findings, enough information will be available for a report. The findings already at hand show that these tumors arise de novo from ductal epithelium, usually in terminal buds, and not from hyperplastic alveolar nodules (HAN), as do spontaneous mammary carcinomas associated with the mammary tumor virus (MTV). In the final part of Dr. Orlando's study he will try to determine whether complete virus replication occurs in mammary duct epithelium, whether viral infection of the mammary mesenchyme occurs, and whether neoplastic mammary epithelium shows evidence of producing virus particles.

(4) A review of tumors of the salivary glands, lacrimal glands, nasal fossa, and maxillary sinuses in mice has been completed in response to an invitation from the editors of the series of publications on Pathology of Tumours in Laboratory Animals.

Proposed Course: Data on the comparisons between PV tumors induced in vivo and PV tumors resulting from PV-transformation of isolated salivary epithelium in vitro are nearly complete, with exception of some of the chromosome studies. It is planned to complete this work and submit a paper for publication in the coming year.

Attempts to clone the culture cell lines that produce tumors showing multi-directional differentiation are underway. It is hoped to obtain perhaps a dozen cloned lines and to determine from the tumors they yield whether the multi-directional differentiation reflects mixed populations in the uncloned lines, or the ability of single stem cells to differentiate in two or more directions.

We now have a large amount of data on the ability of PV tumor tissue to induce/or support growth and morphogenesis of salivary epithelium. This data will be reviewed and additional data acquired to complete a detailed report.

While completion of the above work is in progress, a new phase of the project will be entered. Dr. Suzanne Fisher has begun 2 projects, one aimed toward isolating and identifying one or more factors in PV tumors responsible for induction or support of growth and morphogenesis of normal embryonic salivary epithelium. The other is a comparative study of isozymes in PV tumors and in normal salivary epithelium, as a means of identifying changes related to viral transformational events. The latter study has not yet started, although Dr. Fisher is purchasing the necessary equipment in a major retooling effort. The initial phases of her work will be described in next year's report.

Publications

1. Dawe, C.J.: Tumors of major and minor salivary glands, lacrimal glands, nasal fossa, and maxillary sinuses. In: Turusov, V.S. (Ed.): Pathology of Tumours in Laboratory Animals; Vol. II, Part 1 Tumours of the Mouse. IARC Publication No. 7. (in press)

2. Berman, J., Stoner, G., Dawe, C.J., Rice, J., and Kingsbury, E.: Histochemical demonstration of collagen fibers in ascorbic acid-fed cell cultures. In Vitro (in press)

3. Sakakura, T., Nishizuka, Y., and Dawe, C.J.: Embryogenetic potentials of tissues from adult organs: I. Capacity of adult mammary fat pad to interact inductively with embryonic mammary epithelium. Dev. Biol. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00538-13 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Phylogenetic Aspects of Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.J. Dawe	Head, Comparative Oncology Section - Med. Officer (Path.)	LP NCI
OTHER:	W.G. Banfield	Medical Officer - Pathology	LP NCI
	J.J. Peng	Chief, Cytogenetic Oncology Section	M NCI
	C.M. Poore	Biologist	FCRC NCI
	J.D. Small	Veterinarian	R VR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a continuing study in comparative oncology, designed to identify and characterize neoplasms in lower animals including cold-blooded vertebrates and the invertebrates. By studying neoplasms in animals phylogenetically more remotely removed from man, it is hoped to gain a more complete understanding of the basic biology of neoplasia at all phylogenetic levels. It is expected that such studies will reveal properties of neoplasia not previously suspected to exist because of the excessively narrow scope of studies limited to the higher animals and man. Important differences in the immunologic, biochemical, and regenerative characteristics of the lower animals should serve to emphasize the relevance of these characteristics to the development of and the susceptibility to neoplasia. In addition, by study of neoplasms in lower animals under feral conditions, these neoplasms may be found to serve as indicators of environmental tumorigenic agents. Neoplasms are collected, either in the field or through submittal from outside sources, and are studied morphologically and biologically. Light and electron microscopy, tissue culture, transplantation, and tumor induction methods are applied.

Project Description

Objectives: Viewing neoplastic diseases as a group of biological phenomena that probably affect metazoans at all phylogenetic levels, selected problems have been chosen for investigation, based on availability of tumor material and relevance to research needs concerned with man and other mammals.

A. Cell culture studies of neoplasms in cold-blooded vertebrates are in progress. Objectives are to determine (a) whether neoplastic cells of these animals are able to grow continuously in cell cultures in standard mammalian growth media; (b) whether such cell cultures retain tumorigenic properties; (c) whether such cell cultures retain differentiative properties of the cell of origin; (d) whether viruses are present in the cultured cells; (e) whether the cell lines can be transplanted to allogeneic and xenogeneic hosts; and (f) whether it may be possible to produce immunologic responses against these tumor cell lines in mammalian hosts that might indicate antigens specific for differentiative cell type but non-specific for species of origin of the tumors.

B. Studies of certain tumors (papillomas of pleuronectid fishes and pseudobranch tumors of Pacific gray cod) are being done to determine the nature of as yet unidentified "X cells" in these "tumors". At present it is suspected that X cells are parasitic in nature and that the "tumors" containing them are not neoplasms by most definitions of the term.

Methods Employed:

A. When living specimens of tumor-bearing animals are presented, usually from outside sources such as zoos and fish hatcheries, portions of tumor tissue are taken either at biopsy or necropsy and placed in culture, using standard methods. Cultures are kept at room temperature instead of at temperatures used for mammalian or avian cells. Outgrowths of cells are observed and colonies with features that match those of the neoplastic cell type by histologic criteria are selected for continued culture. When the selected colonies show capability of continuous growth, serial subcultures are carried out and samples of cells are examined microscopically and by transmission electron microscopy. The cell type is characterized structurally and viruses or other microorganisms are looked for. Established cell lines are inoculated into recipients of the same species, into embryonated eggs of the same species, or into athymic mice in the hope of obtaining transplantable tumor lines.

B. Until now, studies of X cells in pleuronectid papillomas and codfish pseudobranch tumors have been purely morphological, using light and electron microscopes and histo- and cytochemical methods. Dr. Herman and Dr. Howley have agreed to apply flow fluorometric methods and DNA characterization techniques to the solution of this problem, if properly collected and prepared tissue material can be provided.

Major Findings:

A. Cells isolated from 3 different neoplasms of poikilotherms are under investigation with results as follows:

1. Chondrosarcoma of corn snake, Elaphe guttatta. The primary tumor was in an albino corn snake in the Baltimore Zoo. Cultures have been maintained continuously for 7 1/2 years. Cartilage matrix is not produced in culture. Electron-dense intracytoplasmic particles were present in primary explants, but are not present in current cultures. Inoculation of culture cells into embryonated corn snake eggs did not give rise to tumors. Inoculations into athymic mice are currently being done. No virus has been identified by EM. The line grows well in Eagle's minimal essential medium supplemented with fetal bovine serum, 10%.

2. Nephroblastoma of trout. The primary tumor was in a rainbow trout, Salmo gairdneri, from the Albert Powell state fish hatchery, Hagerstown, Maryland. Cultures have been maintained for 5 years in Eagle's MEM supplemented with fetal bovine serum, 10%. They have a mixed epithelial and mesenchymal appearance, but do not form tubules and tubule formation is not induced by placing mouse anterior spinal cord (a standard inducer) in contact with cells in culture. However, desmosomes between epithelium-like culture cells are common. This line, like cell lines derived from normal rainbow trout cells, does not survive temperatures above 70°C. No virus has been found by EM.

3. Metastasizing neoplasm (chromatophoroma (?), liposarcoma (?)) of corn snake. This tumor in a pet corn snake, Elaphe guttata, was placed in culture only 1 month ago. The tumor cells are multiplying in culture but it is too early to designate this a cell line. The tumor, an external, ulcerating growth involving a large area 5-10 cm behind the head, is diagnostically problematic. The differential diagnosis lies between non-pigmented chromatophoroma, liposarcoma, and soft tissue sarcoma of other type, poorly differentiated. Multiple metastases were found in liver and lungs at necropsy. The tumor was highly cellular, composed of fusiform and round cells with large nucleoli and "lacey" cytoplasm. In cultures, some tumor cells contain yellow to brownish intracytoplasmic droplets or granules, which may be either lipids or pteridine pigments. EM, histo- and cytochemical studies, and fluorescence studies are in progress, in efforts to classify the tumor more accurately.

B. X cell "tumors" of pleuronectid fishes and Pacific codfish. The morphological studies by light and electron microscopy were completed and published this year (see publications list). Dr. S.R. Wellings, our collaborator at the University of California, Davis, has been unable to collect further specimen material and as a consequence this project is at a standstill.

Significance to Biomedical Research and the Program of the Institute: Study A. The cell culture studies thus far indicate that, like mammalian neoplastic cells, neoplastic cells of poikilotherms are able to adapt to and grow under standard culture conditions. This is not surprising. Since we have not found suitable recipients to determine tumorigenicity of the lines as yet, we have no information on the effects of culture on the differentiative features of these lines. Perhaps the chief value of the lines at present would be for propagation of fish and snake viruses. Usefulness of these lines for this purpose has not been explored.

Proposed Course: We hope to test the tumorigenicity of the trout nephroblastoma line by inoculating cells into kidneys of rainbow trout. These attempts will be contingent on collaboration from trout hatchery biologists, as we have no aquarium facilities. The corn snake chondrosarcoma will be tested for tumorigenicity in athymic mice, as this cell line is able to survive at higher temperature than trout cells. Excessively high cell doses will be used to favor takes.

The more recently isolated snake tumor will be further studied to characterize the cell type, and when the line appears well established it will be tested for tumorigenicity in athymic mice.

Publications

1. Alpers, C.E., McCain, B.B., Myers, M., Wellings, S.R., Poore, M., Bagshaw, J., and Dawe, C.J.: Pathologic anatomy of pseudobranch tumors in Pacific cod, *Gadus macrocephalus*. *J. Natl. Cancer Inst*. 59: 377-398, 1977.

2. Dawe, C.J.: Neoplasms of fish. In chapter entitled *Tumors*, by R.A. Squire, D.G. Goodman, T. Frederickson, M.H. Levitt, C.H. Lingeman, M. Valerio, J.D. Strandberg, J.C. Harshbarger, and C.J. Dawe, in book entitled: *Pathology of Laboratory Animals*, ed. by K. Benirschke, F.M. Gardner and T.C. Jones, Springer-Verlag, New York. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00540-03 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Contractile Tissues in Organ Cultures of Mouse Submandibular Glands

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.D. Morgan	Biologist	LP NCI
OTHER:	C.J. Dawe	Head, Comparative Oncology Section (Med. Officer-Path.)	LP NCI
	C.W. Lee	Biologist	LP NCI
	J.E. Williams	Biologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 4	PROFESSIONAL: 1	OTHER: 3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using time-lapse photographic techniques we have identified three types of contractions in mouse submandibular gland rudiments in organotypic culture. The first and second types caused central and peripheral portions of the gland to contract synchronously. The third type caused the gland to contract asynchronously. Each type of contraction was given a functional name. The first type, which had a latency period that ranged from 30 minutes to 6 hours and appeared to purge the ducts and acini of their contents, is called voiding contraction. The second type, which had a latency period that ranged from 4 to 60 minutes and appeared to force the contents of distal tubules toward main ducts, is called priming contraction. The third type, which had a latency period that ranged from 5 to 14 minutes and appeared to whip acini randomly in a twitching manner, is called churning contraction. A preliminary film of these contractions was presented at the 28th Annual Meeting of the Tissue Culture Association at New Orleans in June, 1977. Subsequently, a documented film of contractions in mouse submandibular gland, as revealed by time-lapse photography, will be completed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project will be 1) to produce a documentary film that clearly shows contractions in organ cultures of mouse embryo submandibular glands; 2) to determine whether the contractile element(s) observed in mouse submandibular glands appear to be myoepithelium or smooth muscle; and 3) to determine whether parasympathetic nerve elements in these glands affect contractions observed there.

Methods Employed: Organ cultures of submandibular glands of 14 day embryo mice will be cultured on glass in modified Rose tissue culture chambers and in tube cultures. Chamber cultures will be photographed, using techniques of time-lapse photography. Tube cultures will be studied by histochemical and electron microscopic techniques. Glands with and without their submandibular ganglia will be studied. Time-lapse photography will be used to make a permanent record of contractions; while histochemistry and electron microscopy will be used to identify components of myoepithelium, smooth muscle, and parasympathetic nerve.

Major Findings: Contractions in organ cultures of 14 day embryo mouse submandibular glands began approximately 6 days after start of culture and continued for more than 30 days. Time-lapse photography show the contractions to be mixed: some appeared synchronous and occurred frequently, and others appeared asynchronous and occurred infrequently.

Significance to Biomedical Research and the Program of the Institute: Contractile cells such as myoepithelium play a significant roll in some tumors of salivary glands (pleomorphic adenomas or so-called mixed tumors, for example). A documentary film on the action of these cells in normal glands may increase our understanding of the role played by contractile cells in neoplasms of salivary glands.

Proposed Course: A manuscript entitled "Microcinematographic Observations of Synchronous and Asynchronous Contractions in Mouse Submandibular Gland Rudiments in Organotypic Culture" will be submitted to In Vitro in June, 1978. Additionally, a documentary film entitled "Synchronous and Asynchronous Contractions in Mouse Submandibular Gland Rudiments in Organotypic Culture" is being produced and will be completed during the period covered in this report.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00560-12 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Electron Probe and Scanning Electron Microscope

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.G. Banfield	Medical Officer - Pathology	LP NCI
OTHER:	C.W. Lee	Biologist	LP NCI
	W.A. Hagins	M.D.	LCP NIAMDD

COOPERATING UNITS (if any)

NIAMDD

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this work is to develop the instrumentation and techniques so that the x-ray and other analytical capabilities of the scanning electron microscope can be applied effectively to biological problems. The subjects of present interest are elastin and stratified squamous epithelium.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop the instrumentation and techniques necessary to apply the electron probe and scanning electron microscope to the analysis of biological specimens.

Major Findings: 1. The ETEC scanning electron microscope-electron probe has been moved to NIAMDD space secured by Dr. Hagins in Building 6, Room SB202. This is a basement room almost free of vibration and electrical interference and close to a machine shop and electrical shop, an ideal situation for the instrument.

2. A wave length dispersive spectrometer with the necessary detector and electrical readouts has been ordered.

3. The transmitted light microscope stage is being altered to allow for both scanning electron microscopy and x-ray analysis.

Significance to Biomedical Research and the Program of the Institute: 1. The elimination of vibration and electrical interference will greatly improve the optical performance of the instrument.

2. The wave length dispersive spectrometer will allow for separation of peaks only several electron volts apart.

3. The stage alteration will allow for x-ray analysis of structures viewed with both the light microscope and the scanning electron microscope.

Proposed Course: 1. Upgrade the Nova 1210 computer in the EDAX system to 32K of memory by the addition of 24K of memory and incorporate a Data General Diskette subsystem.

2. Adapt a new version of the NBS program FRAM to the PDP10 and as soon as the EDAX system is upgraded put it on the Nova 1210 computer.

3. Interface the wave length dispersive spectrometer with the computer for control, data acquisition and reduction.

4. Interface the small computers with the PDP10.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00561-08 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Collaborative Research and Other Activities

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.F. Stanton	Medical Officer - Research	LP NCI

COOPERATING UNITS (if any)

See below

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fifteen percent of the normal work week is spent in the following activities: 1. Member, Smoking and Health Review Board-OD; 2. Member, Asbestos Protocol Subcommittee of the HEW Committee on Coordinated Toxicological Research and Related Programs; 3. Project Officer, NCI, DCBD-NO1-CB-33887, Small Animal Holding Facility; 4. Project Officer, NCI, DCBD-NO1-CB-43912, Hematoxylin Substitute Development; 5. Co-Project Officer, NCI, DCBD-NO1-CB-33874, Registry, Tumors in Lower Animals; 6. Member, Editorial Board Monograph Series, IARC-WHO, Lyon, France; 7. Member, ILARS Subcommittee on Tumors in Laboratory Animals, NRC; 8. Dept. of Commerce, Nat. Bureau of Standards Working Group on Establishment of Definitions and Criteria for Mineral Fibers; 9. Associate Editor, Journal of Environmental Pathology & Toxicology; 10. Associate Editor, Journal of Comparative Pathology. These activities primarily involve investigations into the carcinogenicity of asbestos, other durable fibers, and tobacco products. Additionally they involve the collection of data on neoplasms in laboratory and histochemical investigations as an aid to diagnosis.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00593-23 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

An Investigation of the Collagenous Connective Tissues and Elastin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.G. Banfield	Medical Officer - Pathology	LP NCI
OTHER:	C.W. Lee	Biologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Comparative Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 4	PROFESSIONAL: 3	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Forty-eight samples of neck skin for senile elastotic changes and abdominal skin for controls have been collected and preserved by freezing, and by alcohol and formalin fixation to be used in an electron probe induced x-ray analysis of the changed elastin in senile elastosis.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To elucidate the normal and abnormal structure of elastin and collagen and their associated cellular and intercellular components.

Major Findings: Paraffin embedded and epon embedded specimens are not satisfactory for analysis of elastic fibers in the electron probe.

Significance to Biomedical Research and the Program of the Institute: At the minimum, unembedded tissue such as frozen sections must be used.

Proposed Course: Apply the scanning electron microscope with the energy dispersive x-ray analyzer to the analysis of connective tissues, elastin and elastotic degeneration (senile elastosis) in particular.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00533-05 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on Oncogenic DNA Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.C. Costa	Expert	LP NCI
OTHER:	A.S. Rabson	Director, DCBD - Pathologist	LP NCI
	Y. Nakamura	Visiting Fellow	LP NCI
	U. Thorgeirsson	IPA - George Wash. Univ.	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 5	PROFESSIONAL: 2	OTHER: 3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The general scope of this project is to study biological properties of human herpesviruses and human papovaviruses tentatively associated with tumors in human patients. At present the following topics are under investigation. 1) Studies on the Fc receptor induced by herpes simplex virus; 2) Characterization of the interaction of papovavirus with cells in vitro, and in vivo models.

PHS-6040
(Rev. 10-76)

Project Description

1. a) Continue the characterization of the Fc receptor induced by HSV. At present it appears possible to co-immunoprecipitate viral induced proteins with antigen antibody complexes. The binding of viral protein is saturable and is dependent on the presence of the Fc region of the antibody molecule. Biochemical characterization of the Fc binding molecule will be possible. (U. Thorgeirsson).

b) Assays have been developed to screen for IgG resistant and sensitive strains of HSV. One IgG resistant strain is also resistant to the effect of HSV antibody in plaque assays. (C. Yee).

c) The topology of Fc receptor has been determined by immunofluorescence studies. It appears to be admixed with other viral antigens. (Y. Nakamura).

d) It has been shown that bound IgG and immune complexes internalize. Quantitation of internalization will be undertaken. (T. Tralka, Y. Nakamura, C. Yee).

e) The effects of HSV infection upon the cell membrane are being studied by fluorescent labeling of the cell membrane. (C. Yee, D. Zopf, W. Stewart).

2. The biological properties of different papovaviruses and their DNA *in vivo* are being studied. This includes K virus DNA, different SV40 DNA preparations and SV40 mutants.

Proposed Course: 1. Binding of immunoglobulin and internalization will be quantitated. 2. The effect of IgG on HSV DNA synthesis in different strains will be compared. 3. The effect of HSV infection upon the cell membrane will be studied using fluorescent membrane labeling procedures developed in our laboratory in collaboration with Dr. Zopf and Dr. Stewart (NIAMDD). 4. The effect of EBV infection on Fc receptors will be studied in collaboration with Dr. G. Klein.

Publications

1. Costa, J.C., Rabson, A.S., Yee, C.L., and Tralka, T.S.: Immunoglobulin binding to herpes virus-induced Fc receptors inhibits virus growth. *Nature* 269: 251-252, 1977.

2. Costa, J.C., Yee, C.L., and Rabson, A.S.: Absence of papovavirus T antibody in patients with malignancies. *Lancet* II: No. 8040, 709, 1977.

3. Costa, J.C., Yee, C.L., Nakamura, Y., and Rabson, A.S.: Characteristics of the Fc receptor induced by herpes simplex virus. *Intervirology* 10: 32-39, 1978.

4. Nakamura, Y., Costa, J.C., Tralka, T.S., Yee, C.L., and Rabson, A.S.: Histochemical studies of the Fc receptor induced by herpes simplex virus. *J. Immunol.* (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00534-04 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Structure and Assembly of Virulent and Vaccine Polioviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: N.A. Young	Head, Viral Oncology and Molecular Path. Section (Medical Director)	LP NCI

COOPERATING UNITS (if any)

James Nakano, Enterovirus Unit, Center for Disease Control, Atlanta, Georgia; Dr. Bennett Elisberg and Dr. James H. Vickers, Bureau of Biologics, FDA

LAB/BRANCH

Laboratory of Pathology

SECTION

Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1/3	2/3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Viruses isolated from the gut of a child fed type 1 poliovaccine are demonstrably more neurovirulent for monkeys than reference vaccine regardless of whether the isolated virus strains had undergone mutations in the largest virion polypeptide, VP1.

Similar mutations in polypeptides have been detected following feeding of type 2 poliovaccine. The possible relation of these mutations to neurovirulence is being investigated.

PHS-6040
(Rev. 10-76)

Project Description

The relevance of the polypeptide marker (see previous annual report) to neurovirulence of type 1 poliovirus was assessed by the following experiments. Two clones of virus were isolated from the stool 14 days after feeding LSc2ab vaccine to a normal child. One clone had an unstable VP1 (designated VP1 minus) while the other was representative of the mutant vaccine virus with stable VP1 (designated VP1 plus mutant). Each clone was inoculated into 29 rhesus monkeys. The VP1 minus clone produced paralysis in 6 monkeys with a histologic severity ratio of 8/29 and spread ratio of 5/29. The VP1 plus mutant paralyzed 9 monkeys with a severity ratio of 11/29 and a spread ratio of 5/29. These results indicate that both the VP1 plus and VP1 minus clones isolated 14 days after vaccine feeding are significantly ($p = 0.000018$) and $p = 0.003$ respectively) more neurovirulent than reference LSc2ab vaccine poliovirus but not significantly different from each other. We conclude that the polypeptide marker is not an absolute correlate of the presence or absence of neurovirulence. Further studies are in progress to define the relationship of the polypeptide marker to previously known markers, such as rct 40 and the antigenic marker (Wecker analysis).

Preliminary work with polypeptide profiles of type 2 poliovirus strains indicate that it is not possible to reliably differentiate the Sabin vaccine strain from all wild strains. However, virus strains recovered from the gut and central nervous system of a case of vaccine-associated paralytic disease in an immunodeficient child had polypeptide profiles which differed from reference type 2 poliovirus vaccine. These results are consistent with the hypothesis that vaccine mutations rather than host factors alone may be responsible for the paralysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00535-03 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biologic and Virologic Characterization of Breast Cancer Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N.A. Young	Head, Viral Oncology and Molecular Path. Section (Medical Director)	LP NCI
OTHER:			
	J.A. Strauchen	Surgeon	LP NCI
	S.K. Arya	IPA (Roswell Park Mem. Inst.)	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2/3	2/3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continued efforts to detect evidence of RNA tumor virus in human breast carcinoma cell lines have focused on a search for nucleotide sequences in these cells related to the genomes of mouse mammary tumor virus (MMTV) and Mason Pfizer monkey virus (MPMV). Technical difficulties in the preparation of satisfactory radioactive cDNA probes for these viruses have prevented significant progress in the past 9 months.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00537-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Characterization of the Murine Papovavirus K

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Surgeon	LP NCI
OTHER:	M.F. Law	Visiting Fellow	LP NCI
	K.K. Takemoto	Head, Cellular Virology Section	LVD NIAID

COOPERATING UNITS (if any)

Laboratory of Viral Diseases, NIAID

LAB/BRANCH

Laboratory of Pathology

SECTION

Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

K virus is a papovavirus related immunologically to SV40 and polyoma, known oncogenic papovaviruses. The purpose of the study is to evaluate K virus biologically in tissue culture, characterize the disease states it is responsible for in infection of the mouse, and characterize the virus biochemically.

Project Description

Objectives: Characterization of the nucleic acid of this virus and evaluate its relationship to other known papovaviruses.

Methods Employed: Restriction endonuclease analysis, hybridization techniques, and immunologic relationships by immunofluorescence.

Major Findings: We have constructed a physical map of this genome. By hybridization techniques we have mapped regions of the genome conserved between K virus and polyoma; and between K virus and the primate papovaviruses.

Significance to Biomedical Research and the Program of the Institute: The papovaviruses are known to be oncogenic in laboratory animals. Two of these viruses JC and SV40 have been associated with progressive multifocal leukoencephalopathy in man and monkeys. An evaluation of these viruses from a comparative view point is critical.

Proposed Course: Further evaluation of the genome of K virus will include mapping the site for the initiation of DNA synthesis, evaluation of the transcriptional patterns of the virus, and characterizing the protein coded for by the virus.

Publications

One publication has been submitted and two others are in preparation concerning this virus.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00542-01 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on Oncogenic Primate DNA Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Surgeon	LP NCI
OTHER:	M.F. Law	Visiting Fellow	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work involves the characterization of the human papovaviruses BK and JC, as well as the other primate papovaviruses SV40, SA12, and STMV.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Characterization of these viruses biologically and biochemically. Concerned with identifying functions necessary for oncogenic transformation.

Methods Employed: Nucleic acid hybridization, restriction endonucleases, and tissue culture.

Major Findings: 1) Quantitated amount of viral nucleic acid present in BK transformed hamster cells; 2) Identified a new primate papovavirus isolate (HD) as STMV; 3) Developed new technique for analyzing regions of homology among related DNAs.

Significance to Biomedical Research and the Program of the Institute: Provides further understanding of these oncogenic viruses.

Proposed Course: Intend to apply new hybridization techniques to the human papilloma viruses and the human herpesviruses.

Publications

1. Howley, P.M. and Martin, M.A.: Uniform representation of the human papovavirus BK genome in transformed hamster cells. J. Virol. 23: 205-208, 1977.

2. Holmberg, C.A., Gribble, D.H., Takemoto, K.K., Howley, P.M., Espana, C., and Osburn, B.I.: Isolation of simian virus 40 from rhesus monkeys (*Macaca mulatta*) with spontaneous progressive multifocal leukoencephalopathy. J. Infect. Dis. 136: 593-596, 1977.

3. Khoury, G., Lai, C.-J., Solomon, D., Israel, M., and Howley, P.M.: The human papovaviruses and their potential role in human diseases. Cold Spring Harbor Symposium - Origins of Human Cancer. Cold Spring Harbor, New York, 1977, pp. 971-988.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00517-37 LP

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Report from the Pathological Technology Section

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.J. Coolidge	Head, Path. Tech. Sec. (Supv. Bio. Lab. Tech. (Micro))	LP NCI
OTHER:	L.B. Thomas	Chief, Laboratory of Pathology (Med. Dir.)	LP NCI
	C.J. Dawe	Head, Comparative Oncology Section (Med. Officer (Path.))	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Pathological Technology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9	0	9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition develops and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.

PHS-6040
(Rev. 10-76)

October 1, 1977 - September 30, 1978

Number of investigators	107
Number of pieces of tissue	28,736
Number of bottles of tissue	9,960
Number of blocks cut	17,539
Number of blocks cut serially	1,066
Number of frozen blocks cut	232
Number of blocks recut	2,162
Number of slides stained H & E	29,692
Number os slides stained special	4,780
Number of unstained slides	15,378
Number of slides, H & E, special and unstained	49,850

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBD, NCI
October 1, 1977 through September 30, 1978

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and host's response to these diseases. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1000 patients are seen in consultation each year). The senior staff of the Branch includes the Branch Chief, 7 Senior Investigators, 2 Experts, 2 I.P.A., 5 Clinical Associates, 3 Visiting Fellows and 2 Guest Workers. The Dermatology Branch has made significant contributions to the objectives of the National Cancer Plan. The main research achievements of the Dermatology Branch for the past year are as follows:

Therapy of Skin Cancer and Disorders of Keratinization:

(1) We have continued our studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer and diseases of keratinization.
(2) Eighty-one patients with treatment-resistant disorders of keratinization were treated with oral 13-cis retinoic acid, a synthetic stereoisomer of retinoic acid, for one to 55 weeks. Excellent responses have been observed in patients with congenital ichthyosiform erythroderma, lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris, and cystic acne. However, patients with epidermolytic hyperkeratosis have responded only partially, and patients with psoriasis, nevus comedonicus, X-linked ichthyosis, and Netherton's syndrome have either not responded or have worsened. Ten patients with the nevoid basal cell carcinoma syndrome improved considerably with marked reduction in size of skin tumors after therapy with oral 13-cis retinoic acid. We have more recently been using another derivative of retinoic acid, RO-10-9359, an ethyl ester of a trimethylmethoxyphenyl derivative of retinoic acid. Results with this compound seem to be better than with the 13-cis-retinoic acid especially with regard to epidermolytic hyperkeratosis. Commonly observed side effects included cheilitis, facial dermatitis, xerosis, skin fragility, and conjunctivitis. The mechanism by which these synthetic retinoid alters these disease states is not clear but may be related to the observed ability of vitamin A to affect glycoprotein synthesis and epithelial differentiation.
(3) The profound beneficial effect of 13-cis retinoic acid in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelia, could be successfully treated with the synthetic retinoids. These patients, who have successfully responded to 13-cis retinoic acid, may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Studies of DNA Repair in Normal Human Cells and in Cells from Xeroderma Pigmentosum Patients

(1) UV-radiation is believed to be the major cause of the most common type

of human cancer, cancer of the skin. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor develop in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of other diseases in which DNA repair may be defective, especially those associated with an unusually high incidence of cancer such as ataxia telangiectasia will of course further increase our understanding of the relationships between DNA repair processes and carcinogenesis. XP patients' sun-exposed skin ages much more rapidly than normal human's skin. Some XP patients develop neurological abnormalities which are due to the early death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes and these aging phenomena in XP. Thus, our studies of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans.

(2) We have studied post-UV colony forming ability (CFA) in irradiated XP fibroblasts from each of the six genetic forms of XP. A striking correlation between the degree of reduction in post-UV CFA and the presence and severity of XP-associated neurological abnormalities has been found. These results constitute the first strong evidence for a relationship between the effective level of DNA repair and the severity of a heredo-degenerative condition of the nervous system. These results are relevant to the theory of "intrinsic mutagenesis" which hypothesizes that error-proneness in key DNA repair or replication enzymes is responsible for carcinogenesis and for a variety of degenerative diseases as well as for normal aging in various human tissues. We have confirmed recent brief reports that cells from patients with Cockayne's syndrome, a syndrome consisting of cachectic dwarfism, neurological abnormalities and photosensitivity, have decreased post-UV CFA. In addition, we have found that Cockayne's syndrome patients have a normal pressure hydrocephalus which is probably the cause of some of their neurological abnormalities. Studies in our lab have confirmed reports of decreased post-X-ray CFA in cells from patients with ataxia telangiectasia, a disease in which patients have an increased rate of cancer, plus degenerative findings in the immune and nervous systems. Such studies are now being performed in parallel with post-UV CFA assays on XP cells, Cockayne cells, and cells from patients with other photosensitive and or degenerative disorders.

(3) Studies of UV-induced sister-chromatid exchanges have been performed using normal, XP and Cockayne's syndrome lymphocyte cell lines and have shown that UV-induced sister chromatid exchanges are greater in most XP lymphocyte cell lines than in normal lines. Studies are also being conducted on XP, Cockayne and ataxia telangiectasia cells to determine if these cells have reduced host-cell-reactivation of irradiated purified herpes simplex DNA. Attempts are being made to accomplish in vitro malignant transformation of XP and normal fibroblasts by UV light. If successful, the results of such work

when correlated with our knowledge of DNA repair efficiency and UV mutability, should yield important new information concerning the interrelationships of DNA repair, mutagenesis and carcinogenesis.

Virologic Studies:

(1) Warts represent a viral induced tumor of man. A closely related virus, the Shope papilloma virus of rabbits, will induce benign tumors in some rabbits and malignant tumor in others. The squamous cell cancers of patients with epidermodysplasia verruciformis may be induced by wart viruses, as may tumors of other patients. Methods are being developed which should not only permit the screening of tumors for wart viral material, but also facilitate attempts to propagate the virus in tissue culture. Assuming that the analogy with the Shope papilloma virus is correct, propagation of the virus in tissue culture would facilitate the investigation of the molecular mechanisms which result in a benign proliferation of cells in some instances and malignant transformation in others.

(2) In addition, virologic studies show that an untransformed cloned mink cell which contains the Moloney murine sarcoma viral genome will undergo transformation on treatment with physiologic concentrations of dexamethasone or other glycocorticoids. The extent of transformation correlates with the levels of sarcoma specific viral RNA and with viral p30 protein. Transformation depends on the continued presence of glycocorticoid, since its removal results in loss of transformation and a decrease in viral expression to pretreatment levels.

(3) A sensitive quantitative 10 day transfection assay for murine sarcoma and leukemia viruses has been developed for use with unintegrated and integrated viral DNA. The efficiency of transfection depends on several critical parameters: size of infectious DNA, size of carrier DNA, concentration of DNA, cell density, ph of precipitation medium, and the use of dimethylsulfoxide. With this technique, DNA can be titrated almost as easily as intact virus.

(4) Several mouse teratoma cell lines have been examined for their ability to be infected by a variety of viruses. In the undifferentiated state, these cells were permissive for the replication of all viruses tested except for murine leukemia viruses. After differentiation, a cell line became permissive for Moloney leukemia virus. Nucleic acid hybridization studies indicate that the block to viral expression occurs after synthesis of proviral DNA.

(5) The spleen focus-forming virus (SFFV) has acquired specific xenotropic viral genetic information not contained in Friend helper virus. A cDNA probe that represents the xenotropic virus portion of SFFV detects genetic sequences derived from the env gene region of murine xenotropic virus. SFFV therefore represents a recombinant between ecotropic and xenotropic viral genetic information.

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases:

(1) Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis are blistering skin diseases all of which are associated with so-called autoimmune phenomena. We have identified and characterized serum and in vivo bound antibodies in these diseases. Our efforts have

centered around the mechanisms of tissue destruction, ultrastructure, and ultrastructural localization of antibodies. Gastrointestinal abnormalities, similar to those seen in gluten sensitive enteropathy, occur in dermatitis herpetiformis and are under active study. We have demonstrated that gluten withdrawal from the diet indeed reverses the skin disease in many patients. Immunogenetic considerations with regard to HLA associations and the identification of specific B-lymphocyte antigens have provided tools to study the triggering mechanisms involved in the early stages of an abnormal immune response. Another major effort is the study of the effect of sulfones on neutrophilic exudative disorders. As sulfones dramatically alleviate dermatitis herpetiformis and erythema elevatum diutinum we are using animal models of inflammation and serum and blister fluids from patients to study their effect on complement activation, deposition and neutrophilic chemotaxis.

(2) Several chemically and genetically distinct collagens are found in mammalian species. Types I and III collagens are widely distributed, type II collagen is found only in cartilagenous structures, and type IV is found in basement membranes. Our studies, utilizing light and ultrastructural immunocytochemical techniques have demonstrated that the sera of patients with relapsing polychondritis, a disease characterized by widespread destruction of cartilagenous tissues, contain antibodies with specificity for type II collagen. Additionally we have demonstrated that both Type IV collagen and the non-collagenous basement membrane glycoprotein are localized exclusively to the basal lamina of skin basement membrane.

(3) Mammalian epidermis is composed of three cell types; the keratinocyte whose function is in producing keratin, the melanocyte which produces pigment, and the Langerhans cell (LC) the function of which is unknown. We have demonstrated that human, guinea pig, and mouse epidermis contains a small (2-6%) subpopulation of LC. The cells have Fc and C3 receptors and thereby resemble cells of the histiocyte-monocyte-macrophage series. We have also demonstrated that these cells bear immune response gene associated (Ia) surface markers. Additionally we have demonstrated that these cells can stimulate a mixed leukocyte reaction and can present antigen to sensitized T cells, thereby reinforcing the analogy to monocytes and macrophages.

Studies on Mechanisms of Cellular Immunity in Man:

(1) Our combined in vitro and in vivo controlled methods for the study of cell-mediated immunity in man, have allowed us to initiate basic immunopharmacology and immunogenetics approaches for the study of a variety of diseases and has already resulted in several practical diagnostic procedures. Additional studies promise a method for selecting effective immunotherapeutic agents against specific cancers.

(2) In collaboration with the Diabetes Branch we have discovered a new form of diabetes with a defect in the affinity of insulin-receptors. Preliminary evidence suggests that this defect may relate to circulating plasma factors.

(3) A method has been developed (clonal priming) for defining specificity and cross reactivity at the T cell level. The clonal priming reaction offers a practical, ethical, in vitro, quantitative approach for systematic study of relationships recognized as foreign by immune cells. Concepts of

tolerance and immunohomeostasis have been well developed. However, progress has been hampered by lack of an experimental approach to directly compare tissue, differentiation, tumour, microbial (including viral) and other environmental antigens at the level of cellular immunity. With clonal priming, it may now be possible to pinpoint agents implicated in a variety of inflammatory and neoplastic diseases in which cellular immune discrimination plays a role.

(4) We have found that the allostimulatory ability of human spermatozoa is resistent to ultraviolet (UV) light in striking contrast to the exquisite UV sensitivity of leukocytes.

(5) We have also detected cytotoxic lymphocytes which are specific for dinitrophenylated autologous leukocytes.

Biochemical Characterization of Mammalian Melanosomes:

(1) The abnormal proteins produced in malignant melanoma are being studied: these studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential.

(2) In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of isoelectric points (4.8 and 4.5, respectively). There also is a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids (asp, gly, ala, leu).

(3) The observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. These results indicate that although melanogenesis takes place, its metabolic pattern is abnormal. There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanosomes.

(4) Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in both murine and avian pigmentary systems, but not peroxidase, as has been suggested by some workers. It has always been a subject of dispute how tyrosinase, which can be easily demonstrated to be present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inactivated *in vivo* in mammalian systems and subsequently activated once *in situ* in the melanosome. We have found that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues.

Applications of SEM to Soft Biological Tissues:

SEM reveals fine surface structure with unparalleled three-dimensionality, depth of field, and sampling capability. We have been specifically concerned with the possible influence of the mitotic cycle on the surface morphology of cells in monolayer culture; since neoplastic transformation generally accelerates and alters the cell cycle, any topographic comparisons between nonneoplastic and neoplastic cell populations must be considered in the light of cycle-dependent changes. Previous work from this laboratory has ruled out any major influence of interphase stages on the topography of the commonly used Chinese hamster ovary (CHO) cell line in unperturbed standard monolayer culture, but the impact of mitosis on the surface form of these cells has required the analysis of representative populations of nonsynchronized cells positively identified in each stage of cell division and compared critically with a representative population of authenticated interphase cells. Comparisons among the cell populations in interphase and in each stage of mitosis reveal substantial shifts in the degree of expression of three topographic features, i.e., roundness and the densities of microvilli and blebs.

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments:

(1) The epidermis offers a unique opportunity for the study of tumors not only because of the quantitative importance of this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of the keratin subunits in a well defined cell culture system will facilitate studies of carcinogenesis in vitro. In addition, the finding of large amounts of actin protein in epidermal tumors, and the development of suitable immunoassay procedures for it is of potentially great significance.

(2) The seven polypeptide chains which comprise the subunits of the keratin filaments of normal bovine epidermis have been isolated and individually characterized by standard protein chemical techniques. The subunits polymerize in vitro into native-type filaments. The repeating structural unit of the filaments consists of three chains aligned side-by-side with two discrete regions of super-coiled α-helix interspersed by regions of non helix. These regions have been isolated by selective digestion with trypsin and appear in two forms. The structure of the basic repeating unit of the epidermal keratin filament is being pursued using ultrastructural techniques. Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. Using purified keratin proteins, a specific radioimmunoassay has been developed for immunocytochemical purposes. The synthesis of the keratin filament subunits by the mouse epidermal cells implies the presence within the cells of the complete protein biosynthetic apparatus, and this system is therefore potentially very useful for studying control of differentiation in the epidermis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03630-08 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Morphologic and Biochemical Effects of Vitamin A and Analogs on Chick and Human Skin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
OTHER:	P.V. Bhat	Visiting Fellow	Derm NCI
	P.M. Steinert	Expert Consultant, Biochemist	Derm NCI
	B.K. Wetzel	Senior Investigator	Derm NCI
	L. DeLuca	Senior Investigator	Exp. Path, NCI
	P.M. Elias	Asst. Professor, Dept. of Dermatology Univ. of California Medical Center	

COOPERATING UNITS (if any)

Experimental Pathology Branch

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.29	PROFESSIONAL: 1.29	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project proposed to morphologically and biochemically define the mechanism of action of vitamin A and its derivatives (retinoids) in altering epidermal differentiation in embryonic skin, normal skin, and in benign and malignant lesions of skin.

Chick metatarsal skin, when cultured in vitro, is stimulated to undergo mucous metaplasia by vitamin A acid. Ultrastructural analysis alone cannot determine whether a post-translational effect, a genomic effect, or an effect of vitamin A acid on the mesenchyme is the primary mechanism involved in the inhibition of keratinization and inducation of epidermal mucin biosynthesis by vitamin A acid.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To morphologically investigate the mechanism by which vitamin A alters epidermal cell differentiation with resultant inhibition of keratinization in embryonic skin, normal adult skin, and in benign and malignant lesions.

3) To study the early changes of vitamin A induced mucous metaplasia at the ultrastructural level. In particular, to determine if epidermal cells are capable of both mucin and keratin production, or whether the two processes are mutually exclusive.

4) To define certain dermatologic disorders of keratinization at the biochemical level.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

1) Metatarsal shank skin (devoid of feathers) is obtained from 13-14 day chick embryo.

2) 0.2-0.4 mm thick, Castroviejo keratotome slices of normal and diseased human skin. Skin from patients with Ichthyosis and Darier's Disease (NCI-3643) has been used to date.

3) 3-4 mm punch biopsies of normal and diseased human skin (NCI-3643).

Methods Employed:

Chick metatarsal skin is cultured in vitro and stimulated to undergo mucous metaplasia by vitamin A acid. Specimens are evaluated for mucin production by the use of special stains, such as PAS (with and without diastase), Alcian Blue, Toluidine Blue, and Colloidal iron. Specimens are also examined under the transmission and scanning electron microscopes. In addition, the scanning electron microscope is being used to study in ovo skin scale and feather development, and their inhibition to vitamin A acid in vitro.

Major Findings:

The influence of vitamin A on differentiating epithelia was examined in explants of skin from 14-day chick embryos exposed to retinoic acid (RA) in low, moderate, and high doses. The changes observed in RA-treated cultures are both dose- and time-dependent and are reversible when explants are transferred to control medium. The periderm sloughs prematurely and horizontal stratification is lost. Keratinization is inhibited and fewer desmosomes and tonofilaments are seen. Surface epidermal cells develop microvilli, bulge upwards, and detach. Golgi elements, rough endoplasmic reticulum, and polyribosomes are unusually prominent. Mucin granules form and gland-like structures develop with intercellular canaliculi characterized by tight junctions, brush borders, and dense secretory contents.

On the basis of present evidence there are several possible mechanisms by which RA could alter epidermal differentiation. RA-induced gaps in the basal lamina allow direct contact between epidermal basal cells and fibroblasts and collagen fibers which could result in inappropriate dermal signals reaching the epidermis. In younger embryos the entire epidermis, including the mitotically inactive surface cells, appears to respond to RA, and this could imply an epigenetic modulation of cell phenotype. Finally, after the formation of a stratum corneum in older embryos only the relatively undifferentiated basal layer shows a metaplastic response, indicating that RA could be acting directly on the genome.

2) Preliminary electrophoretic analyses have indicated that several major protein components found in normal skin are lacking in involved skin in Darier's. This data, might indicate that either certain metabolic processes are inhibited in patients or that excessive degradation of the protein components has occurred. Further work is being done to resolve this issue in Darier's disease and other cutaneous disorders of keratinization.

3) In search of active metabolites of retinol and retinoic acid, these agents were used to treat spontaneously transformed mouse fibroblasts in cell culture experiments- with exposure to these agents, the fibroblasts exhibited increased cell-cell and cell-substrate adhesion but decreased saturation density. One metabolite of retinoic acid synthesized by these cells had the chromatographic properties of retinyl phosphate. Alkaline hydrolysis of this product yielded anhydroretinol, which is also the hydrolysis product of retinly phosphate. Two metabolites of retinol were synthesized by the fibroblasts. One was identified as anhydroretinol and the other as an ester of retinol, which is probably an intermediate in the formation of anhydroretinol.

Significance to Cancer Research:

1) Since carcinogenesis is an instance of altered differentiation, studies of vitamin A-induced redifferentiation, both in human and chick skin, may serve as an excellent model for investigations of cellular

control mechanisms which relate to carcinogenesis. The fact that carcinogen metabolism and carcinogenesis are influenced very markedly by vitamin A deficiency directly links research on vitamin A epithelial effects to cancer research.

2) Furthermore vitamin A is of value in the treatment of malignancy. The mechanisms of action of vitamin A in inhibiting keratinization may be related to its antineoplastic activity.

Proposed Course of Project:

1) Continued ultrastructural examination of the above material.

2) The effect of vitamin A and its derivatives on the glycosyltransferase activities of normal and diseased skin will be studied.

3) Further attempts will be made to characterize the epidermal proteins in cutaneous disorders of keratinization.

Publications:

1) Peck, G.L., Elias, P.M., Wetzel, B.: Effects of retinoic acid on embryonic chick skin. J. Invest. Dermatol. 69: 463-476, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03632-08 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cutaneous Manifestations of Graft-Versus-Host Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
OTHER:	T.J. Lawley	Clinical Associate	Derm NCI
	H.M. Moutsoupoulos	Visiting Fellow	NIAMDD
	A.B. Deisseroth	Section Head	PO NCI

COOPERATING UNITS (if any)
Experimental Hematology Section, Pediatric Oncology Branch, NCI
Laboratory of Microbiology & Immunology, NIDR

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.01	.01	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing clinical study of patients with acute and chronic Graft-Versus-Host Disease (GVHD) has led to the discovery of one patient with acute GVHD whose cutaneous eruption progressed to Toxic Epidermal Necrolysis and another patient with chronic GVHD who developed cutaneous scleroderma and a sjogren's like syndrome. Histological and ultrastructural studies are being done on these patients and on other current patients with GVHD.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the pathogenesis of the cutaneous manifestations of Graft-Versus-Host Disease (GVHD) particularly Toxic Epidermal Necrolysis (TEN), scleroderma and sjogren's syndrome and to investigate the relationship between them.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone and in combinations) in the cure and palliation of cancer.

Methods Employed:

1) Examination of patients with aplastic anemia or acute lymphocytic leukemia, treated with allogeneic bone marrow transplantation, who developed GVH with skin manifestations.

2) Examination with the electron microscope of multiple skin biopsies taken at various time intervals and at various skin sites.

Major Findings:

The cutaneous manifestations of acute GVHD are initially morbilliform and, subsequently, scarlatiniform.

1) Clinical Findings: One case was observed in which the initial morbilliform eruption of GVHD progressed to sheet-like desquamation of the epidermis, typical of TEN. Another patient with chronic GVHD developed sclerodermatous skin and xerophthalmia and xerostomia resembling sjogren's syndrome.

2) Electron microscopic findings: The initial pathologic events in GVHD in the patient who developed TEN in the basal layer of the epidermis, and it appears that the mononuclear infiltrate may play a significant pathogenetic role. Mitochondrial swelling, coarsening of tonofibrils and increased prominence of ribosomes were noted in all basal cells. The nuclei appeared contracted, with more convoluted nuclear membranes; increased density of chromatin was noted. Lymphocytes were occasionally seen within the basal layer, but only rarely above. Increased numbers of Odland bodies and abnormal keratohyalin granules were noted in the superficial epidermis.

Significance to Cancer Research:

1) Immunosuppressed patients, including treated cancer patients, who are transfused or transplanted with immunocompetent cells, may expect

to develop GVHD. In view of our experience, we may now expect some of them to develop TEN, cutaneous sclerosis, or a Sjogren's-like syndrome as well.

Proposed Course of Project:

Electron microscopic examination of specimens from other patients with skin manifestations of chronic GVHD are being studied.

Publications:

1) Lawley, T.J., Peck, G.L., Moutsopoulos, H.M., Gratwohl, A.A., Deisseroth, A: Scleroderma and a sjogren's like syndrome in a patient with chronic graft-vs-host disease. Ann. Int. Medicine 87:707-709, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03638-09 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of DNA Repair in Normal Human Cells and in Cells from Xeroderma Pigmentosum Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Robbins	Senior Investigator	Derm NCI
OTHER:	A.N. Moshell	Expert	Derm NCI
	S.A. Newfield	Clinical Associate	Derm NCI
	K.I. Snowden	Gnotobiotics Unit Head	VRB DRS
	S. Potkay	Chief, Small Animal Section	VRB DRS
	D.R. Lowy	Senior Investigator	Derm NCI
	R.J. Goldberg	Senior Investigator	VOP NCI
	R.E. Tarone	Mathematical Statistician	B NCI
	J. Whang-Peng	Medical Director	MC NCI

COOPERATING UNITS (if any)

Medicine Branch, Clinical Oncology Program, DCT, NCI, Small Animal Section, VRB, DRS: Biometry Branch, DCCP, NCI; Viral Oncology Program, DCCP, NCI.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 7.5	PROFESSIONAL: 3.9	OTHER: 3.6

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies in this laboratory are designed to elucidate the role of DNA repair processes in human diseases, carcinogenesis, and ageing. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP), all of whom have defective DNA repair plus the clinical abnormalities of premature ageing of the skin and cutaneous malignancies. We measure the DNA excision repair process by assays of UV-induced unscheduled DNA synthesis. We assess the biological effectiveness of DNA repair with assays of post-irradiation colony-forming ability and trypan blue exclusion and have found a striking correlation between the level of effectiveness of DNA repair in XP and the severity of XP-associated neurological abnormalities. We have studied the relationship of UV-induced sister chromatid exchanges to the repair defects of XP. We are extending our work to include cells from patients with other diseases in which DNA repair defects are claimed or suspected, such as Cockayne's syndrome, ataxia telangiectasia, Fanconi's anemia, Werner's syndrome, Rothmund Thompson syndrome and progeria. We have established lymphocyte cell lines (long-term lymphoblast lines) from all six genetic forms of XP. These lines are useful in radiation-sensitivity and biochemical tests.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study DNA repair processes in normal cells, cells from patients with each of the six genetic forms of xeroderma pigmentosum (XP), and cells from patients with other diseases in which defective DNA repair is claimed or suspected; to determine the relationships between the function of such DNA repair processes and the clinical findings of photosensitivity, premature aging, and skin or other cancers.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Materials:

Cells are currently obtained from established cell repositories. They will also be obtained from hospitalized patients and outpatients at the NIH and occasionally as gifts from other investigators. Cells currently under study are dermal fibroblasts and lymphocyte cell lines (long-term lymphoblast lines).

Methods Employed:

Rates of excisional DNA repair are determined by using scintillation spectroscopy or autoradiography to measure the UV-induced unscheduled incorporation of tritiated thymidine into X-ray or ultraviolet (UV)-irradiated cells. The biological effectiveness of repair is tested by assays of post-irradiation colony-forming ability (CFA) and by post-irradiation trypan blue viability tests. Sister chromatid exchange, before and after irradiation, was determined by Hoechst staining and examination of cells in the appropriate metaphase; we have analyzed the results obtained. Cells are cloned, and the cloned cells are tested in post-irradiation colony-forming tests to determine the origins and bases for experimental results. Photobiological tests in vitro, similar to those used with XP cells, are being conducted on cultured cells from patients with ataxia telangiectasia and patients with Cockayne's syndrome. XP and normal human cells are being irradiated and injected into nude mice to determine if radiation-induced malignant tumors will develop. Our very sensitive autoradiographic techniques for determining the uptake of tritiated thymidine into DNA is being employed to detect mycoplasma contamination of human cells in tissue culture.

Major Findings:

1) We have studied post-UV colony-forming ability (CFA) in irradiated XP fibroblasts from each of the six genetic forms of XP. A striking

correlation between the degree of reduction in post-UV CFA and the presence, onset and severity of XP-associated neurological abnormalities has been found. These results constitute the first strong evidence for a relationship between the effective level of DNA repair and the severity of a heredo-degenerative condition of the nervous system.

2) The post-UV CFA studies of certain XP strains have revealed a somewhat greater UV resistance at high UV doses than at low UV doses. Cloning experiments are in progress to determine if such results are due to the presence of two distinct cell populations or are a relfection of dose-dependent differences in repair efficiency intrinsic to all the cells in these strains

3) We have confirmed recent brief reports that cells from patients with Cockayne's syndrome, a syndrome consisting of cachectic dwarfism, neurological abnormalities and photosensitivity, have decreased post-UV CFA. Our studies on cells from nine unrelated Cockayne's syndrome patients have revealed that there are significant differences among Cockayne strains in the degree of reduction in post-UV CFA. No defects in UV-induced unscheduled DNA synthesis have been found, however, in any of these strains.

4) We have previously completed a clinical study of patients with Cockayne's syndrome. Analysis of all abnormal clinical findings in such studies have given us an important opportunity to relate the clinical manifestations of this syndrome to the laboratory findings we have done with XP patients. We have found that Cockayne's syndrome patients have a normal pressure hydrocephalus which is probably the cause of some of their neurological abnormalities.

5) Studies in our laboratory have confirmed reports of decreased post-X-ray CFA in cells from patients with ataxia telangiectasia, a disease in which patients have an increased rate of cancer, plus degenerative findings in the immune and nervous systems. Such studies are now being performed in parallel with post-UV CFA assays on XP cells, Cockayne cells, and cells from patients with other photosensitive and or degenerative disorders.

6) We have succeeded in establishing a total of 25 lymphocyte cell lines (long-term lymphoblast lines) from the six genetic forms of XP. Work to date with several such lines indicates that they retain the same level of diminished DNA repair as the fresh peripheral lymphocytes from which they arose and that they have decreased post-UV survival. Further characterizations of all such lines is underway. These lines should be valuable in attempts to characterize and purify the defective DNA repair enzymes in XP. Such lines have also been established from patients with Cockayne's syndrome and ataxia telangiectasia.

7) In collaboration with Dr. Weng-Chow Cheng and Dr. Jacqueline Whang-Peng of the NCI, studies of UV-induced sister-chromatid exchanges have

been performed using normal, XP and Cockayne's syndrome lymphocyte cell lines. UV-induced sister chromatid exchanges are greater in most XP lymphocyte cell lines than in normal lines, and the results have been analyzed with the assistance of Dr. R.E. Tarone.

8) Studies in co-operation with Drs. Robert Goldberger and Douglas Lowy of the NCI are being conducted on XP, Cockayne and ataxia telangiectasia cells to determine if these cells have reduced host-cell-reactivation of irradiated purified herpes simplex DNA.

9) Attempts are being made to accomplish in vitro malignant transformation of XP and normal fibroblasts by UV light. The irradiated cells are injected into nude mice to determine if malignant tumors will form. If successful, the results of such work, when correlated with our knowledge of DNA repair efficiency and UV mutability, should yield important new information concerning the interrelationships of DNA repair, mutagenesis and carcinogenesis.

10) Autoradiographic studies of XP cells after UV irradiation have shown that a very small (ca 1% of normal) but detectable amount of UV-induced unscheduled DNA synthesis occurs in cells from typical patients in complementation group A, the genetic form of XP having the least such synthesis. This work has demonstrated the great sensitivity of our assay system; no other laboratory has been able to measure such small amounts of unscheduled DNA synthesis.

11) Autoradiographic screening of cells for the presence of mycoplasma (detected as 'cytoplasmic' incorporation of tritiated thymidine) has been shown to be of great value, especially when dealing with those species of mycoplasma not easily detected by standard culturing techniques. Such screening is of great importance to all work on DNA repair as mycoplasma contamination can significantly alter the results of repair assays and, if undetected, could lead to erroneous conclusions. The sensitivity of our detection method is being compared to standard fluorescent and culture techniques for detecting mycoplasma.

Significance to Biomedical Research and the Program of the Institute:

UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor develop in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of other diseases in which DNA repair may be defective, especially those associated with an unusually high incidence of cancer such as ataxia telangiectasia will further increase our understanding of the relationships between DNA repair processes and carcinogenesis.

XP patients' sun-exposed skin ages much more rapidly than normal human's skin. Some XP patients develop neurological abnormalities which are due to the early death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes and these aging phenomena in XP. Thus, our studies of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

1) Petinga, R.A., Andrews, A.D., Tarone, R.E., and Robbins, J.H.: Typical xeroderma pigmentosum complementation group A fibroblasts have detectable ultraviolet light-induced unscheduled DNA synthesis. Biochim. Biophys. Acta 479:400-410, 1977.

2) Andrews, A.D., Barrett, S.F., and Robbins, J.H.: Xeroderma pigmentosum neurological abnormalities correlate with post-ultraviolet light colony-forming ability. Proc. Nat. Acad. Sci. USA., in press, 1978.

3) Andrews, A.D., Barrett, S.F., Yoder, F.W., and Robbins, J.H.: Cockayne's syndrome fibroblasts have increased sensitivity to ultraviolet light but normal rates of unscheduled DNA synthesis. J. Invest. Dermatol. in press, 1978.

4) Brumback, R.A., Yoder, F.W., Andrews, A.D., Peck, G.L., and Robbins, J.H.: Normal pressure hydrocephalus: recognition and relationship to neurological abnormalities in Cockayne's syndrome. Arch. Neurol., in press, 1978.

5) Andrews, A.D., Barrett, S.F. and Robbins, J.H.: The relationship between neurologic disease, acute sun sensitivity and post-ultraviolet colony-forming ability in xeroderma pigmentosum. In DNA Repair Mechanisms (Hanawalt, P.C., Friedberg, E.C., and Fox, C.F., Eds.). Academic Press, New York, in press, 1978.

6) Robbins, J.H., Andrews, A.D., and Moshell, A.N.: DNA repair nucleotide sequences which prevent premature death of neurons in humans: evidence from studies on xeroderma pigmentosum (Hanawalt, P.C., Friedberg, E.C., and Fox, C.F., Eds.). Academic Press, New York, in press, 1978.

7) Robbins, J.H.: Workshop Summary: Xeroderma Pigmentosum. In DNA Repair Mechanisms (Hanawalt, P.C., Friedberg, E.C. and Fox, C.F., Eds.). Academic Press, New York, in press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03647-05 D

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Scanning and Transmission Electron Microscopy of Leukocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. K. Wetzel	Senior Investigator	Derm NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.02	0.02	0.04

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To define the surface ultrastructure of leukocytes under a wide range of conditions and thereby provide fresh insight into the physiology of normal and leukemic cells; also, to characterize definitively the origin, contents and fate of the characteristic granules of neutrophils, eosinophils, and basophils as an index of the development, function and fate of these cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To define the surface ultrastructure of leukocytes under a wide range of conditions and thereby provide fresh insight into the physiology of normal and leukemic cells; also, to characterize definitively the origin, contents, and fate of the characteristic granules of neutrophils, eosinophils, and basophils as an index of the development, function and fate of these cells.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Investigate the biology of cells already capable of forming cancer to determine the relationship of their growth and interaction with the host response.

Methods Employed:

Cells from whole blood have been fixed directly by bleeding into a large volume of fixative, collected on filters, stained, freeze-dried, and examined by SEM. Leukocytes found in the central area of the filter were mapped, and micrographed, then relocated, identified and photomicrographed by light microscopy.

Major Findings:

The leukocytes examined in this way were nearly all spherical and covered with microvilli, as noted previously in this laboratory; lymphocytes, neutrophils and monocytes were represented in very small numbers, and neither eosinophils nor basophils were found in our sample. The method is extremely tedious and inefficient and not suited for broader studies withour further modification.

Significance to Cancer Research:

The reactions of lymphocytes and macrophages to immune responses in malignant growth and therapy are largely dependent on cell surface phenomena and cell interaction -- both uniquely amenable to study by SEM and ancillary methods.

A thorough understanding of the origin and nature of leukocyte granules is necessary to define and interpret the differentiation of normal granulocytes, the cytopathology of chronic granulocytic leukemia and related disorders, and to help clarify the nature and functions of granulocytic infiltrates in skin and other tissues in malignant diseases.

Proposed Course of Project:

Pending modification of our methods, we will be engaged in a thorough description of the topography of peripheral leukocytes from normal humans obtained in representative yields. This approach will be extended to studies of hematopoiesis and the leukemias. A major study of leukocyte granulogenesis is planned employing quantitative autoradiography, cytochemistry and TEM. Light microscopic monitoring of preparative conditions and the dynamics of leukocyte adherence, motility, interaction, and growth will be continued with the use of thin perfusion chambers.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03649-06 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Enzymatic Control Mechanisms in Melanogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. J. Hearing	Research Biologist	Derm NCI
OTHER:	K. J. Tomecki	Clinical Associate	Derm NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has partially characterized the nature of tyrosinase, and determined the mechanism of normal latency of the enzyme, and its subsequent activation in vivo. The role of this enzyme in melanogenesis in normal tissue and the importance of the altered enzyme found in melanoma tissues is being studied.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To investigate the control mechanisms involved in regulating melanogenesis in a variety of normal and malignant tissues.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Tyrosinase and other enzymic activities are investigated both by spectrophotometric and radioactive assay methodology. Tissues are dissected immediately before use in 0.1 M phosphate buffer, homogenized and fractionated into subcellular fractions by means of density gradient centrifucation and differential centrifugation. After solubilization with detergents, enzymes are further purified by polyacrylamide gel electrophoresis. Samples are then incubated with the appropriate substrates and controls. The production of melanin and other reaction products can be followed spectrophotometrically or by liquid scintillation counting of the newly formed radioactive melanin or byproducts.

Major Findings:

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in both murine and avian pigmentary systems, but not peroxidase, as has been suggested by some workers. It has always been a subject of dispute how tyrosinase, which can be easily demonstrated to be present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inactivated in vivo in mammalian systems and subsequently activated once in situ in the melanosome. In amphibians, the mechanism seems to be proteolytic activation; in mammalian and avian systems, however, proteolysis has no function in this regard. L-DOPA is the natural activator of the enzyme in vivo; we have tested over twenty L-DOPA analogs substituted in various positions, and determined the enzyme is extremely specific in its requirement for L-DOPA as a cofactor. It has been found in this study that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues.

Significance to Cancer Research:

In view of the differences noted between the regulation of tyrosinase in normal and melanoma tissues, we feel that at lease one of the primary differences between the melanogenic capabilities of these two tissues has been revealed. The fact that these controls are operational at the post-translational level is informative; whether other levels of cellular control mechanisms are affected by carcinogenesis remains to be investigated. It is hoped that further study of the cause of these enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Proposed Course of Project:

Differences in the control mechanisms over melanogenesis in normal and malignant melanoma tissues will continue to be investigated. The characteristics of enzymatic activity and control in various subcellular fractions are being studied. This project has been expanded to include characterization of similar metabolisms in normal and malignant human tissues.

Publications:

1) Hearing, V.J., Ekel, T.M., Montague, P.M., Hearing, E.D., and Nicholson, J.M.: Mammalian tyrosinase: isolation by a simple new procedure, and characterization of its steric requirements for cofactor activity. Arch. Biochem. Biophys. 185:407-418, 1978.

2) Hearing, V.J., Nicholson, J.M., Montague, P.M., Ekel, T.M., and Tomecki, K.J.: Mammalian tyrosinase - structural and functional interrelationship of isozymes. Biochim. Biophys. Acta. 522:327-339, 1978.

3) Eppig, J.J., Jr., and Hearing, V.J.: Melanogenesis in normal and malignant tissues: Effects of trypsin, phospholipase C and Triton X-100 on tyrosinase activity. Pigment Cell (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03650-06 D

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Mammalian Melanosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. J. Hearing	Research Biologist	Derm NCI
OTHER:	K. J. Tomecki	Clinical Associate	Derm NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The abnormal proteins produced in malignant melanoma are being studied: these studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To characterize the structural and enzymatic composition of melanin granules throughout their development in vivo, both in normal and abnormal pigmentary systems.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Our research has been aimed primarily at characterizing the chemical composition of melanosomes in urine and human pigment systems, both in the normal as well as in melanoma tissues. Tissues are excised and homogenized in 0.25M sucrose, and the melanosomes are then isolated and purified by means of an extensive series of differential centrifugations. Granules thus isolated are solubilized in either sodium decyl sulfate, 8 M urea or Triton X-100, then examined with the aid of polyacrylamide gel electrophoresis. Computer analysis of the resulting protein separations allows the calculation of various physiochemical parameters of these macromolecules, including molecular weight, molecular size, valence and several other physical constants that are most useful in the comparisons of various proteins. Preparative polyacrylamide gel electrophoresis has enabled mg quantities of the proteins under study to be isolated in a pure state. This allows further characterization of these proteins with regard to their amino acid content, isoelectric point, molecular weight, and prosthetic group content.

Major Findings:

The melanosome in normal mouse tissue is composed of multiple proteins, many of which are loosely bound and easily extracted. These probably constitute the proteins of the limiting membrane of the organelle. Another protein is of lower molecular weight and is tightly bound to the granule, and probably constitutes the structural, fibrillar protein; it appears to be the protein which complexes with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in melanoma melanosomes are unique and are not found in normal melanin granules. This has been found to be the case in human melanoma as well. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of isoelectric points (4.8 and 4.5, respectively). There also is a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids (asp, gly, ala, leu).

Significance to Cancer Research:

The observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. Melanomas are unusual among cancers since they do not result in complete differentiation of the affected melanocyte, but allow this cell's specialization, i.e. melanogenesis, to continue. These results indicate that although melanogenesis takes place, its metabolic pattern is abnormal.

There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanosomes. An analogous situation of altered proteins has been described in several other types of malignancies; thus this process of atypical formation of proteins may be common to neoplastic transformation. The similarity of these proteins suggests that the unique proteins present in the malignant melanocyte are an aberrant form of the analogous protein in the normal melanocyte, although the mechanism effecting this has not yet been elucidated.

Proposed Course of Project:

Since there are indications that the metabolism of melanogenesis in murine melanoma is aberrant, our research is ultimately intended to examine human melanomas, and attempt to characterize the level on which these disorienting control mechanisms operate. It is hoped that further insight into such controls, be they at the level of replication, transcription, translation, or post-translation, will provide clues as to the level on which carcinogenic information is expressed.

Publications:

1) Nicholson, J.M., Montague, P.M., Ekel, T.M. and Hearing, V.J.: Isolation and partial characterization of aberrant melanosomal proteins from normal and malignant murine melanocytes. Pigment Cell (in press).

2) Hearing, V.J., Kerney, S.E., Montague, P.M., Ekel, T.M. and Nicholson, J.M.: Characterization of the intracellular location of tumor-associated antigens in B-16 murine malignant melanoma. Pigment Cell (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03652-05 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on Mechanisms of Cellular Immunity in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.R. Levis	Senior Investigator	Derm NCI
OTHER:	A.M. Dattner	Instructor, Albert Einstein School of Medicine	IEP AIP
	S.K. Shama	Clinical Associate	Derm NCI

COOPERATING UNITS (if any)

Diabetes Branch, NIAMD, Immunology Branch, DCBD, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	3.0	3.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A better understanding of cell mediated immunity in man. Particularly, normal or abnormal immunologic mechanisms involved in epithelial and lymphoid malignancies, auto-immune disease, allograft rejection and allergic contact dermatitis. Characterization of the abnormal blastogenic responses found in certain diseases, particularly lymphoid malignancies. The necessity of both lymphocytes and macrophages in blastogenic responses in vitro has been elucidated and the lymphocyte has been identified as the cell which determines the immunologic specificity of the blastogenic response to antigens. A soluble mediator, released by macrophages, has been found to be an essential requirement for the blastogenic response to antigens and foreign tissues. Soluble mediators (lymphokines), released by various subpopulation of lymphocytes, have also been detected and studied at varying times following spefically induced cell-mediated contact sensitivity reactions. Our combined in vitro and in vivo controlled methods for the study of cell-mediated immunity in man, have allowed us to initiate basic immunopharmacology and immunogenetics approaches for the study of a variety of diseases and has already resulted in several practical diagnostic procedures. Additional studies promise a method for selecting effective immunotherapeutic agents against specific cancers.

Project Description:

Objectives:

1) To investigate the mechanisms concerned with the control of mitosis, differentiation, function, and potentialities of normal and pathologic cells, with particular reference to the lymphocyte and cells of the lymphoreticular system.

2) To investigate the functional and morphological interactions between lymphocytes and macrophages and among different populations of lymphocytes during *in vitro* immune responses related to delayed hypersensitivity, homograft reactions, and cell mediated allergic contact dermatitis.

3) To detect, characterize, and isolate biologically active substances, including soluble mediators of cellular immunity, produced in stimulated and unstimulated human leukocyte cultures. To study their effects modulation, and differentiation *in vitro*.

4) To develop an *in vitro* system for studying allergic contact dermatitis in humans including a method of monitoring immunotherapy of skin cancers with DNCB.

5) To develop a useful and sensitive histocompatibility test in humans.

6) To develop an *in vitro* model for the study of homograft rejection in humans.

7) To develop an *in vitro* system for studying immune reactivity to various skin cancers.

8) To determine the efficacy of 2,4 dinitrochlorobenzene (DNCB) on cutaneous malignancies and premalignant growth in subjects sensitized to DNCB.

9) To evaluate DNCB as a useful model for the study of immunotherapy of certain human cancers and possibly lead to additional immunotherapy studies.

10) To develop an *in vitro* model for studying and screening drugs which modulate cell mediated immunity in humans.

The Objective and Approach of the National Cancer Plan that this project most closely suppors is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (along and in combinations) in the cure and palliation of cancer.

Material:

1) Lymphocytes and other peripheral blood cells are obtained from hospitalized patients and outpatients including patients with chronic lymphocytic leukemia, sezary syndrome, and other disease states.

2) Keratatome strips of human skin.

3) Excised tumors of human skin.

4) Sperm cells obtained from human ejaculates.

5) Fibroblasts, lymphoid cells from humans and other species maintained in tissue culture.

Methods Employed:

1) Human peripheral blood lymphocytes are cultured *in vitro* and stimulated to transform into large blastoid cells by factors such as phytohemagglutinin, antigens, and foreign tissue. This phenomenon of blastogenesis is evaluated by the morphologic and biochemical changes which the lymphocytes undergo. The mechanisms by which these blastogenic factors act and the culture conditions required for blastogenesis are determined.

2) Human peripheral blood leukocytes are separated into a glass-adherent cell population, composed largely of macrophages, and into a population consisting of small lymphocytes. By recombining such populations obtained from the same and from different individuals, the function of each population is determined. Interactions and contacts between cells are examined by light and scanning electron microscopic techniques, including methods for observing the same cells by both modalities.

3) A method has been developed for freeze storing leukocytes for subsequent studies of the blastogenic response to various antigens and mitogens.

4) A method has been developed for adapting the dinitrochlorobenzene contact sensitization test to the *in vitro* lymphocyte transformation system.

5) Methods have also been developed for freeze storing human spermatozoa with preservation of HLA-D (LD) antigens.

Major Findings:

1) Methods for obtaining 'purified' lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human

peripheral blood have been developed and analyzed. We have improved and utilized a very rapid and simple 5-minute centrifugation method using heparinized whole blood for obtaining a population of mononuclear leukocytes (lymphocytes and monocytes) with very few contaminating granulocytes. This mononuclear leukocyte population contains the two cell types required for the blastogenic response and provides cultures capable of responding with maximal responses to specific and non-specific blastogenic factors.

2) Human epidermal cells induce blastogenesis in allogeneic leukocytes. Human epidermal cells did not induce blastogenesis in autologous leukocytes nor in the leukocytes of a mixed leukocyte HL-A identical sibling pair. These results indicate that epidermal induced leukocyte blastogenesis is a measure of histocompatibility in humans comparable to a one-way mixed leukocyte reaction.

3) A method has been developed which permits positive light microscopic identification of individual leukocytes in a heterogeneous population by Giemsa staining while preserving delicate surface features for subsequent scanning electron microscopy of the same cells.

4) Isolation and partial characterization of a factor released by glass adherent phagocytic cells which is an essential requirement for the blastogenic response of lymphocytes to specific antigens, leukocytes, and phytohemagglutinin.

5) Isolation of a factor released in cultures containing lymphocytes of subjects sensitized to DNCB that will induce blastogenesis in leukocyte cultures from nonsensitized subjects.

6) Characterization of the abnormal blastogenic responses of lymphocytes from patients with chronic lymphocytic leukemia, sezary syndrome, and other related disease states.

7) Development of a method for monitoring the degree of sensitivity to DNCB _in vitro_ during in vivo contact sensitization. This _in vitro_ technique expands the usefulness of DNCB for the evaluation and study of cell mediated immunity, and offers a method for monitoring DNCB immunotherapy, an approach for the study of tolerance, and a controlled system for the study of the many antigen-released mediators of cellular immunity in man. Similar approaches with other contact allergens may offer a safe diagnostic test for allergic contact dermatitis.

8) Successful adaptation of the Dinitrochlorobenzene (DNCB) contact sensitization test to the _in vitro_ lymphocyte transformation technique in humans.

9) Development of simplified methods for freeze storing human peripheral blood leukocytes.

10) Using the frozen leukocyte technology and the method for studying DNCB sensitivity *in vitro* we have demonstrated a temporal relationship between *in vivo* skin reactivity and *in vitro* specific lymphocyte transformation during a primary immune response.

11) Peak in vitro activity was observed two to four weeks following application of the primary sensitizing dose of DNCB and fell off to lower levels within two months of primary sensitization. These sequential studies of allergic contact dermatitis in humans are the first such studies of the kinetics of a primary cellular immune response using both *in vivo* and *in vitro* techniques.

12) Additional studies have demonstrated a quantitative relationship between the intensity and duration of allergic contact dermatitis to DNCB *in vivo* and the magnitude of specific lymphocyte transformation *in vitro*.

13) Demonstration that repeated challenge with DNCB boosts specific in vitro lymphocyte transformation.

14) Discovery of lymphokine activity in allergic contact dermatitis there is evidence that such lymphokines may be the mediators of cell mediated immunity.

15) Demonstration of the time course of release of lymphokine activity in human peripheral blood cultures.

16) Demonstration of reproducible dose-response curves for lymphokine activity. Such dose-response curves illustrate a sensitive quantitative assay for cellular immunity *in vitro* and offers a biological system with great potential for further characterization and isolating the mediators in cell mediated immunity.

17) The DNCB system offered the versatility that made it possible to show the lymphokines responsible for lymphocyte transformation in target leukocyte populations from nonsensitized subjects were as active on syngeneic (identical twins) leukocytes as on allogeneic leukocytes, indicating the observed activity was not due to histocompatibility factors.

18) It has recently been shown that lymphokine activity is absent during a primary immune response to DNCB and required one or more rechallenges before activity is detectable using the lymphocyte transformation system which suggests the soluble factors known as lymphokines, may represent an amplification of cellular immunity.

19) Successful adaptation of an automated microsystem for harvesting cells has enabled us to harvest cells in a rapid fashion.

20) The unique features of this project are: a) Both *in vitro* and

in vivo portions of this project are ethically feasible in humans which makes these studies more germane to human disease. b) Previous studies with microbial antigens have lacked effective control over exposure, in contrast to controlled DNCB sensitization. c) The assay is sensitive, specific and quantitative making it a valuable tool for measuring lymphocyte activation under a variety of experimental conditions.

21) Studies on soluble factors released by human lymphocytes (lymphokines) indicate that little or no lymphokine is released during an in vivo primary immune response to DNCB, but is produced in some subjects only after additional in vivo rechallenge. We interpret this to indicate that the lymphokine activity demonstrated in vitro may well indicate a bodily mechanism for amplifying the cellular immune response.

22) Recent studies in our laboratory indicate that an entire series of DNP containing amino acids induce varying degrees of lymphocyte transformation specifically in subjects topically sensitized with DNCB. The ability to stimulate with soluble DNP containing substances as well as with our previously developed particulate antigen offers great flexibility in our combined in vitro - in vivo system for investigating the cellular immune response in humans. Furthermore, these studies might prove to be of considerable importance in understanding the mechanism of triggering a specific cell mediated immune reaction.

23) Demonstration of the ability of sperm cells to induce allospecific lymphocyte transformation. We have previously demonstrated the ability of macrophages, certain lymphocytes, and human epidermal cells to induce allospecific lymphocyte transformation. Interestingly, studies in the mouse indicated that a product of the major histocompatibility complex has a tissue distribution similar to the cell populations we have found to induce allospecific lymphocyte transformation in humans. These findings could have important impact in diverse areas such as transplantation and reproductive biology. We are currently attempting to find out if our studies on contact sensitivity are related or in any way controlled by the major histocompatibility complex in humans. The combination of our histocompatibility and contact sensitivity studies may develop into a field of human immunogenetics with potential ramifications in a wide variety of disease states.

24) We have developed methods for freeze-storing human spermatozoa and preserving their ability to stimulate allogeneic lymphocytes. This methodology, which uncovers the important HL-A-D (LD) locus responsible for graft rejection allows a practical and superior approach to study of the genetic locus containing immune response genes associated with a variety of human diseases.

25) In collaboration with the Diabetes Branch we have discovered a new form of diabetes with a defect in the affinity of insulin-receptors.

Preliminary evidence suggests that this defect may relate to circulating plasma factors (Clin. Research - Abstract (in press) Manuscript in preparation).

26) We have developed a method (clonal priming) for defining specificity and cross reactivity at the T cell level. The clonal priming reaction offers a practical, ethical, in vitro, quantitative approach for systematic study of relationships recognized as foreign by immune cells. Concepts of cross reactivity, organ- and tissue-specific antigens, autoimmunity, tolerance and immunohomeostasis have been well developed. However, progress has been hampered by lack of an experimental approach to directly compare tissue, differentiation, tumour, microbial (including viral) and other environmental antigens at the level of cellular immunity. With clonal priming, it may now be possible to pinpoint agents implicated in a variety of inflammatory and neoplastic diseases in which cellular immune discrimination plays a role.

27) Clonal priming has been extended to the study of soluble protein antigens (Dattner and Levis, IRCS #1) which required a method of representation in our second culture (IRCS #2). This method should allow the study of autologous components (immune response gene products) and identification of specific environmental antigens which may be involved in the immunopathogenesis of major histocompatibility (MHC) associated diseases.

28) We have found the leukocyte autologous components (immune response gene products) required for lymphocyte activation in DNCB sensitized subjects are sensitive to ultraviolet light and behave in a manner similar to HLA-D determinants (Levis, Lincoln & Dattner, Fed. Proc. Abstract - manuscript submitted for publication).

29) We have found that the allostimulating ability of human spermatozaa is resistant to ultraviolet (UV) light in striking contrast to the exquisite UV sensitivity of leukocytes. This finding is not only interesting in distinguishing these two measures of histocompatibility, but gives us significant possible insight on the mechanisms involved in recognition processes and provides putative evidence for an effect at the genetic (DNA) level.

30) We have reported the detection in our culture system of cytotoxic lymphocytes which are specific for dinitrophenylated autologous leukocytes. Cytotoxic activity was demonstrated in a 4 hour chromium release assay using unstimulated peripheral blood lymphocytes as targets. Lytic activity was not observed in fresh PBL but was demonstratible by day 5 of primary in vitro stimulation and could be demonstrated as late as day 21. Cytotoxic activity was often markedly increased by secondary in vitro restimulation. Lysis was observed only on DNP-modified cells. Between certain donors total self-restriction of cytotoxic activity was observed (i.e. lysis was observed

on the autologous but not the allogeneic target). Between other donors, partial cross reactivity was observed in the DNP-dependent lysis. This self-specificity is consistent with effector cell recognition of DNP in association with polymorphic cell surface antigens most likely those coded for by HLA-linked genes. Such findings may be significant for understanding the pathogenesis of contact sensitivity, in vitro screening for contact sensitizers, and of potential practical benefit for predicting the immunotherapeutic value of various haptens in the treatment of cancer.

31) In collaboration with Dr. Gary Peck and others in the dermatology branch, we have found a promising method for treatment of some skin cancers with 13-cis retinoic acid.

Significance to Biomedical Research and the Program of the Institute:

The lymphocyte is a cell involved in immune reactions which may be operative in the immune response to neoplastic tissue. Our studies concerned with blastogenesis and the control of cell division and differentiation of the lymphocyte may elucidate the cell's normal and abnormal functions in the immune response involved in the host's defense against cancer. These studies should also provide information concerning the control of cell differentiation and division.

Proposed Course of Project:

Because of some success in the treatment of skin cancer with 13-cis Retinoic Acid and topical dinitrochlorobenzene we plan to escalate our clinical program for the treatment of skin cancer while continuing our basic studies of the mechanisms involved in the human immune response. In this regard a recent report by Dennert et al indicates that Retinoic Acid has an effect on the differentiation of T-cell killers, a finding in keeping with some of our clinical projects on the treatment of patients with extensive skin cancer and our finding of in vitro T-cell killers in contact sensitivity reactions. The finding of an autologous leukocyte product required for lymphocyte activation in DNCB sensitized subjects also offers promise for the treatment of some patients with autoimmune alopecia and a possible rational approach for immunotherapy of skin cancer. If this approach proves rewarding for skin cancer, it should be extendable to other forms of neoplasia. Escalation of our clinical studies should add to our progress in basic understanding of immune mechanisms in man and our goal is to continue a balance between basic and clinical studies.

Publications:

1) Dattner, A.M., Levis, W.R.: Unresponsiveness induced by priming human leukocyte cultures with optimal stimulating concentrations of a soluble protein antigen. IRCS Medical Science 5:342, 1977.

2) Dattner, A.M., Levis, W.R.: Restoration of accelerated blastogenesis to previously unresponsive high dose-PPD. Paralyzed clonally primed lymphocytes. IRCS Medical Science 5:526, 1977.

3) Levis, W.R., Lincoln, P.M., Dattner, A.M.: Effect of UV light on HLA-D of blood leucocytes and sperm. Lancet 1:987, 1978.

4) Bar, R.S., Levis, W.R., Rechler, M.M., Harrison, L.C., Siebert, C., Padskalny, J., Rogh, J., and Muggeo, M.: Extreme insulin resistance in Ataxia Telangiectasia: Defect in affinity of insulin receptors. New Engl. J. Med. 248:1164-1171, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03654-05 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Applications of SEM to Soft Biological Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. K. Wetzel	Senior Investigator	Derm NCI
OTHER:	K. Sanford	Head, Cell Physiology & Oncogenesis	LB NCI
	G. Jones	Microbiologist	LB NCI
	E. Kendig	Biologist	Derm NCI

COOPERATING UNITS (if any)

Laboratory of RNA Tumor Viruses, NCI

LAB/BRANCH
Dermatology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.0	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To discover and develop new methods and interpretive criteria for the application of SEM to a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional, experiential view of cell form and cell surface phenomena, and 3) an unusual potential for cytochemical studies.

Project Description:

Objectives:

To discover and develop new methods and interpretive criteria for the application of SEM to a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional, experimental view of cell form and surface phenomena, and 3) an unusual potential for cytochemical studies.

We have been specifically concerned with the possible influence of the mitotic cycle on the surface morphology of cells in monolayer culture; since neoplastic transformation generally accelerates and alters the cell cycle, any topographic comparisons between nonneoplastic and neoplastic cell populations must be considered in the light of cycle-dependent changes. Previous work from this laboratory has ruled out any major influence of interphase stages on the topography of the commonly used Chinese hamster ovary (CHO) cell line in unperturbed standard monolayer culture, but the impact of mitosis on the surface form of these cells has required the analysis of representative populations of nonsynchronized cells positively identified in each stage of cell division and compared critically with a representative population of authenticated interphase cells.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.5: Study cell surface and cell membranes.

Methods Employed:

Tissue culture cells are grown as monolayers on glass coberslips, fixed by the addition of an equal volume of buffered 6% glutaraldehyde to the medium, rinsed, and stained by the Feulgen procedure. The coverslips are mounted in water, and examined by light microscopy. All cells in a predetermined central area are mapped and photomicrographed to detail nuclear chromatin and chromosome patterns, and all mitotic cells in a larger concentric area are similarly mapped and recorded.

The coverslips are then processed appropriately for SEM. All cells in the central area and all mitotic cells in the larger concentric area are relocated and micrographed by SEM to record topographic features. Each of these cells is scored (0 - 4+) with respect to roundness, and the incidence of microvilli and blebs, and the data from several specimens are tabulated and and analyzed.

Major Findings:

Comparisons among the cell populations in interphase and in each stage of mitosis reveal substantial shifts in the degree of expression of three topographic features, i.e., roundness and the densities of microvilli and blebs.

Interphase cells tend to be relatively flat with moderate numbers of microvilli and few blebs, and range from flat and smooth to fully rounded and villous. Most interphase cells lack blebs but increased numbers appear on moderately rounded, moderately villous cells. Prophase cells generally resemble interphase cells/ they remain relatively flat, but become more blebbed and significantly more villous. At the onset of metaphase, most cells become fully rounded and covered with microvilli with somewhat fewer blebs. Anaphase cells remain similarly rounded and villous with slightly increased blebbing, and elongate as the chromosomes separate. Telophase cells become considerably less round and less villous, most (85%) become blebbed, and the cell assumes a paired configuration during cytokinesis which often persists into interphase.

This study supports the following conclusions concerning CHO cells grown under these conditions: 1) Smooth-surfaced cells are nonmitotic. 2) Approximately 60% of fully rounded cells covered with microvilli are in metaphase or anaphase. 3) Elongated cells are in anaphase. 4) Paired cells with many blebs are probably either in telophase or newly arrived in interphase. 5) Generally, a rigorous sampling of cell populations which have been characterized by independent means is necessary to ensure valid interpretation of SEM images.

Significance to Cancer Research:

SEM reveals fine surface structure with unparalleled three-dimensionality, depth of field, and sampling capability. As this approach is adapted to biological problems through experience with diverse tissues, cellular form, surface details and interactions can be better interpreted in normal and malignant tissues, as well as in experimental systems which help define immune responses, differentiation, and other processes fundamental to oncogenesis and its control. The tissue culture studies directly elucidate new aspects of tumor cell biology and transformation.

The current study documents for the first time substantial shifts in surface form which occur as cells undergo mitosis. More importantly, by sampling systematically and scoring morphologic parameters independently for each cell in the sample, it is possible to analyze representative populations of heterogeneous cells rather than selected nonneoplastic, preneoplastic, and neoplastic cell populations which typically display nonuniform surface morphology.

Proposed Course of Project:

To further develop and refine techniques of specimen preparation, analysis, and interpretation for SEM will be the goal of continuing work with a wide vareity of tissues, experimental approaches, and instrumentation. Special emphasis will be accorded a search for novel cytochemical methods, especially with labels visualized in the backscatter mode, which are uniquely possible for this tool. Further collaboration in SEM, TEM and cytochemical studies of skin is under way with investigators in this branch and elsewhere.

Publications:

1) Wetzel, B., Kendig, E.M., Jones, G.M., and Sanford, K.K.: A systematic scanning electron microscope (SEM) analysis of mitotic cell populations in monolayer culture. SEM, 1978, O. Johari, Ed., p. 1-10.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03656-05 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Steinert	Expert	Derm NCI
OTHER:	G.L. Peck	Senior Investigator	Derm NCI
	W.W. Idler	Chemist	Derm NCI
	S. Yuspa	Section Head	DCCP NCI

COOPERATING UNITS (if any)

Experimental Pathology Branch, DCCP

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The polypeptide chains which comprise the subunits of the keratin filaments of normal human, murine and bovine epidermis have been isolated and characterized. The subunits polymerize in vitro into native-type filaments. The repeating structural unit of the filaments consists of three chains aligned side-by-side with two discrete regions of super-coiled α-helix interspersed by regions of non-helix. Higher orders of filament ultrastructure are being investigated using physiochemical and electron microscopic techniques as well as sequence studies on individual subunits. The keratin polypeptide subunits of abnormal and malignant human epidermis are different from those of normal epidermis and work is in progress to define these differences. Normal mouse epidermal cells grown in cell culture synthesize the keratin filaments. The appearance of these proteins will be used as biochemical markers for studies of in vitro carcinogenesis. An actin-like protein is present in a variety of human and mouse epidermal tumors and its use as a marker for malignant tumors is being investigated.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To characterize the ultrastructure of keratin filaments polymerized in vitro from the subunits of the filaments isolated from normal human, murine and bovine epidermis.

2) To characterize the polypeptide subunits of keratin filaments isolated from various benign and malignant human and mouse epidermal diseases.

3) To investigate the biosynthesis of the keratin filament polypeptide subunits in cultures of normal mouse epidermal cells and to investigate the effects of various drugs and carcinogens on the cells.

4) To characterize the actin-like protein detected and isolated from normal mouse epidermal cells grown in culture and from mouse and human epidermal tumors.

Major Findings and Methods Employed:

1) The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 A wide and up to 40 um long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptide are present in the filaments in the precise molar ratios of 1:2. This suggests that the epidermal keratin filament has a three-chained unit structure and this is supported by the X-ray diffraction analyses.

2) The X-ray diffraction data implies the filaments certain region of coiled-coil α-helix. These regions have been isolated by selective digestion with trypsin and appear in two forms: All of the helix of the filaments can be accounted for by (a) particles 20A X 180A, 95% α-helix, of 40,000 molecular weight, which can be denatured with SDS into chains of about 14,000 molecular weight. Therefore the α-helix of the filaments consists of discrete regions 100A long of triple chain coiled-coil α-helix. (b) particles present during the early stages of digestion are 20A x 400A, 75% α-helix, of 110,000 molecular weight and can be denatured with SDS into chains of about 35,000 molecular weight. End-terminal analyses indicate that this consists of two helical regions of (a) separated by about 40A of non-helix. This fragment contributes the major portion of and contains all of the

α-helix of the triple-chain structural unit of the filaments. The properties and structure of these fragments provide information on the alignment of the polypeptides in the structural unit. The structural unit is 20A wide by about 500A long and consists of these filament subunits aligned side-by-side with two α-helical segments interspersed by regions of non-helix.

3) Having presented a model for the structure of the basic repeating unit of the epidermal keratin filament, further studies on the ultrastructure of the filament have followed two different but complimentary directions. Only the bovine system has been studied because of the adequate availability of protein. (a) Details of the primary structure of selected filament subunits will provide information on how the unit structure forms and aggregate to form the filament. The α-helical particles as well as peptides derived on cleavage of intact subunits with NBS and CNBr are being characterized; the former will be located along the subunits following ordering of the NBS and CNBr peptides. (b) Higher orders of filament structure are being investigated by studying the mechanism of filament assembly. Polymerization is time and protein concentration dependent and follows an ordered biphasic cooperative process. This involves the initial rate-limited formation of a "nuclear" particle followed by a rapid aggregation of it into filaments. The nuclear particle is a hexamer, as determined by mathematical models of light-scattering data, and probably consists of a pair of triple chain structural units and may be a structural "building block" of the filament. In support of this filament assembly appears in the electron microscope to involve (i) formation of 20A wide particles which may correspond to the structural unit and hexamer; (ii) apparent twisting of these to form short filaments about 80A wide; and (iii) apparend end-to-end joining of these to form long filaments. Detailed studies attemptint to define the assembly process and hence filament ultrastructure are underway.

4) Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier's disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Attempts are underway to identify such differences by comparisons of two-dimensional gel electrophoretic maps of the CNBr and NBS peptides of the proteins obtained from normal and abnormal epidermis.

5) Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro

6) The synthesis of the keratin filament subunits by the mouse epidermal cells implies the presence within the cells of the complete protein biosynthetic apparatus, and this system is therefore potentially very useful for studying control of differentiation in the epidermis. This is of importance as there are a number of diseases of the epidermis which may be attributed to disorders of the differentiative process of the tissue. Polyribosomes of the size necessary to synthesize the keratins can be isolated and attempts are underway to isolate the messenger RNA responsible for their synthesis of the proteins.

7) A variety of human and experimentally-induced mouse epidermal tumors (carcinomas and papillomas) also contain large amounts of the actin-like protein. Because this observation has great potential as a marker for malignancies, the actin protein has been isolated and attempts will be made to prepare an antibody for a specific immunofluorescent- or radioimmuno-assay procedure. The large amounts of actin protein should provide a suitable biochemical marker for studies of the malignancies.

Significance to Cancer Research:

The epidermis offers a unique opportunity for the study of tumors not only because of the quantitative importance of this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of the keratin subunits in a well defined cell culture system will facilitate studies of carcinogenesis in vitro. In addition, the finding of large amounts of actin protein in epidermal tumors, and the development of suitable immunoassay procedures for it is of potentially great significance.

Proposed Course of Project:

Studies on the abnormal (benign and malignant) tissues will be done in collaboration with Dr. Gary Peck. The cell culture studies will be done in the Experimental Pathology Branch in collaboration with Dr. Stuart Yuspa. The biochemical, biophysical and electron microscopic studies of the structure of normal bovine epidermal keratin filaments will be done in this laboratory.

Publications:

1) Steinert, P.M. and Yuspa, S.H.: Biochemical evidence for keratinization by mouse epidermal cells in culture. Science (in press), 1978.

2) Steinert, P.M.: Structure of the three-chain unit of the bovine epidermal keratin filament. *J. Mol. Biol.* (in press), 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03657-04 D

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.I. Katz	Senior Investigator	Derm NCI
OTHER:	T.J. Lawley	Clinical Associate	Derm NCI
	G. Stingl	Visiting Fellow	Derm NCI
	K. Tamaki	Visiting Fellow	Derm NCI

COOPERATING UNITS (if any)

Metabolism Branch, NCI, LCI, NIAID

LAB/BRANCH

SECTION
Dermatology Branch

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis are blistering skin diseases all of which are associated with so-called autoimmune phenomena. We have identified and characterized serum and in vivo bound antibodies in these diseases. Our efforts have centered around the mechanisms of tissue destruction, ultrastructure, and ultrastructural localization of antibodies. Gastrointestinal abnormalities, similar to those seen in gluten sensitive enteropathy, occur in dermatitis herpetiformis and are under active study. Immunogenetic considerations with regard to HLA associations and the identification of specific B-lymphocyte antigens have provided tools to study the triggering mechanisms involved in the early stages of an abnormal immune response. Another major effort is the study of the effect of sulfones on neutrophilic exudative disorders. As sulfones dramatically alleviate dermatitis herpetiformis and erythema elevatum diutinum we are using animal models of inflammation and serum and blister fluids from patients to study their effect on complement activation, deposition and neutrophilic chemotaxis. Alterations in collagen synthesis in inflammatory disease and identification and functional characterization of alloantigens on epidermal cells are other integral parts of the overall program.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To investigate the mechanisms involved in the expression of certain immunologic skin diseases, namely pemphigus, bullous pemphigoid, dermatitis herpetiformis and herpes gestationis.

2) To determine the cell surface characteristics of the lymphocytes and epidermal cells involved in these diseases.

3) To determine whether or not genetic factors are important in the developments of these diseases.

4) To determine the ultrastructure and ultrastructural localization of antibodies in blistering skin diseases.

5) To define the physico-chemical characteristics of the antigen which is in antigen-antibody complexes.

6) To determine the functional capabilities of Ia-bearing epidermal cells in mice and guinea pigs.

Material:

Skin biopsies, either punch of Castroviejo keratome slices will be used. Also the small intestine of patients with dermatitis herpetiformis will be studied in order to determine their binding characteristics. Blister fluid studies for inflammatory mediators are also under investigation. Guinea pigs, mice and rabbits are used for the identification of lymphocyte and epidermal cell surface antigens as well as for the production of antibodies.

Methods Employed:

Direct and Indirect Immunofluorescence, Cell Mediated Cytotoxicity, Immunochemical methods for identifying immunoglobulins and immune complexes. Radioimmunoassays. Mixed leukocyte cultures and in vitro antigen priming studies.

Major Findings:

1) All of 51 patients with dermatitis herpetiformis have in vivo bound IgA and various complement components in their skin.

2) The exact location of these antibodies has been sought and found to localize ultrastructurally in 1) clumps in the dermis, 2) associated with anchoring fibrils in the dermis or 3) along the lamina lucida of the basement membrane.

3) The early ultrastructural destruction of the skin in these patients has been demonstrated to correlate well with the antibody localization.

4) Almost all patients with dermatitis herpetiformis have morphologic and enzymatic evidence of gastrointestinal abnormalities very similar to those seen in patients with gluten-sensitive enteropathy.

5) In some patients it is possible to not only reverse the gastrointestinal abnormalities but to reduce or completely eliminate their requirement for sulfones by strict adherence to a gluten-free diet.

6) 85% of 51 patients with immunologically proven dermatitis herpetiformis have HLA-B8 as compared to 20% of controls.

7) All patients with dermatitis herpetiformis have a specific antigen on their B-lymphocytes. This antigen is present in a small percentage of the normal population.

8) Sulfones dramatically benefit patients with erythema elevatum diutinum.

9) By studying twelve patients with herpes gestationis with immunologic methods we have been able to identify the serum factor responsible for deposition complement at the skin basement membrane zone, the site of earliest destruction. Also, by reviewing our own and others reports, it is apparent that herpes gestationis is associated with increased fetal morbidity and mortality.

10) Ultrastructural localization of complement correlates well with the earliest destruction seen in the electron microscope in herpes gestationis and further strengthens the concept that immunologic mechanisms play an important, if not essential, role in the pathophysiology of this disease.

11) The sera of patients with vitiligo associated with multiple endocrinopathies contain antibodies to melanin producing cells.

12) Langerhans cells which comprise a small population of guinea pig epidermal cells, bear C3 and Fc receptors as well as alloantigens which are Ia associated. The Ia antigens can be detected by using a technique which has been refined by us.

13) Langerhans cells have the ability to stimulate a mixed leukocyte reaction and have the capability to present antigen to sensitized lymphocytes.

14) The sera of most patients with Sjogrens syndrome contain large amounts of circulating immune complexes which are distinct from rheumatoid factor.

15) The sera of patients with relapsing polychondritis contain antibodies to type II collagen.

16) Type IV collagen is localized to the basal lamina of skin.

Significance to Cancer Research:

A basic understanding of immunologic injury in various autoimmune disease states is important in interpreting and further current concepts in self-recognition. Pemphigus is associated with thymoma and myasthemia gravis and its study may provide a clue as to the association between pemphigus and other malignancies. Our in depth studies of herpes gestationis, an immunologic blistering disease of pregnancy, may provide important clues to maternal-fetal interactions.

Proposed Course of Project:

Outlined above.

Publications:

1) Hertz, K.C., Crawford, P.S., Chez, R.A., and Katz, S.K.: Herpes gestationis - an update. Obstetric and Gynecology. 49:733-736, 1977.

2) Hertz, K.C., Aaronson, C. and Katz, S.I.: Juvenile dermatitis herpetiformis - an immunologically proven date. Pediatrics 59:945-948, 1977.

3) Katz, S.I., Gallin, J.I., Hertz, K.C., Fauci, A.S., Lawley, T.J.: Erythema elevatum diutinum: Skin and systemic manifestations, immunologic studies, and successful treatment with dapsone. Medicine 56: 443-455, 1977.

4) Katz, S.I.: Dermatitis herpetiformis: Clinical, histologic, therapeutic and laboratory clues. Int. J. Derm. (In press).

5) Hertz, K.C., Gazze, L., Kirkpatrick, C., and Katz, S.I.: Autoimmune vitiligo: Detection of antibodies to melanin-producing cells. New Engl. J. Med. 297: 634-647, 1977.

6) Katz, S.I.: Guest editorial. Histocompatibility antigens (HLA) and disease. Arch. Dermatol. 113:1715, 1977.

7) Yaoita, H., Katz, S.I.: Circulating IgA antibasement membrane zone antibodies in dermatitis herpetiformis. J. Invest. Dermatol. 69: 558-560, 1977.

8) Yaoita, H., Foidart, J.M., Katz, S.I.: Localization of the collagenous component in skin basement membrane. J. Invest. Dermatol. 70:191-193, 1978.

9) Katz, S.I., Strober, W.: The pathogenesis of dermatitis herpetiformis. J. Invest. Dermatol. 70:63-75, 1978.

10) McCarthy, D.M., Katz, S.I., Gazze, L., Waldmann, T.A., Nelson, D.L., Strober, W.: Selective IgA deficiency associated with total villous atrophy of the small intestine and an organ-specific anti-epithelial cell antibody. J. Immunol. 120:932-938, 1978.

11) Stingl, G., Katz, S.I., Shevach, E., Wolff-Schreiner, E., and Green, I.: Detection of Ia antigens on Langerhans cells in guinea pig skin. J. Immunol. 120:570-578, 1978.

12) Stingl, G., Katz, S.I., Abelson, L., Mann, D.L.: Immunofluorescent detection of human B cell alloantigens on S-Ig positive lymphocytes and epidermal Langerhans cells. J. Immunol. 120:661-664, 1978.

13) Kaplan, A.P., Horakova, Z., Katz, S.I.: Assessment of tissue fluid histamine in patients with urticaria. J. Allergy and Clin. Immunol. (In press).

14) Lawley, T.J., Stingl, G., Katz, S.I.: Fetal and maternal risk factors in herpes gestationis. Arch. Derm. 114:552-555, 1978.

15) Katz, S.I.: Recruitment of basophils in delayed hypersensitivity reaction. J. Invest. Dermatol. (In press).

16) Stingl, G., Katz, S.I., Shevach, E.M., Rosenthal, A.S., and Green, I.: Analogous function of macrophages and langerhans cells in the initiation of the immune response. J. Invest. Dermatol. (In press).

17) Provost, T.T., Yaoita, H., Katz, S.I.: Herpes gestationis. In Immunopathology of the Skin, 2nd edition, Beutner, E. et al. (Eds.). (In press).

18) Katz, S.I.: Dermatitis herpetiformis. In Immunopathology of the Skin, 2nd edition, Beutner, et al. (Eds.) (In press).

19) Katz, S.I., Provost, T.T.: Herpes gestationis. In Dermatology in General Medicine, 2nd edition, Fitzpatrick, T.T., et al. (Eds.) (In press).

20) Katz, S.I., Marks, J.: Dermatitis herpetiformis. In Dermatology in General Medicine, 2nd edition, Fitzpatrick, T.B. et al. (Eds). (In press).

21) Katz, S.I.: Dermatitis herpetiformis. In Immunodermatology, R. Good and B. Safai (Eds.) (In press).

22) Moutsopoulos, H.M., Bolow, J.E., Lawley, T.J., Stahl, N.I., Antonovych, T.T., and Chused, T.M.: Immune complex of glomerulonephritis in sicca syndrome. Amer. J. Med. 64:955-069, 1978.

23) Levinson, A.I., Summers, R.J., Lawley, T.J., Evans, R., and Frank, M.M.: Evaluation of the adverse effects of long term hyposensitization. J. Aller. Clin. Immunol. (in press), 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03659-04 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Therapy of Skin Cancer and Disorders of Keratinization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
OTHER:	T.G. Olsen	Clinical Associate	Derm NCI
	M. Pandya	Visiting Fellow	Derm NCI
	D. Butkus	Visiting Fellow	Derm NCI
	J. Arnaud-Battandier	Guest Worker	Derm NCI
	J. Strauss	Professor, Dermatology	Univ. of Iowa
	D.T. Downing	Professor, Dermatology	Univ. of Iowa

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 4.7	PROFESSIONAL: 4.7	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was similarly tested and found to be more effective and less toxic than 13-cis retinoic acid in the treatment of the disorders of keratinization.

Project Description:

Objectives:

To evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne.

Agents:

1) 13-cis retinoic acid, 10 mg and 20 mg capsules
2) An ethyl ester of a trimethylmethoxyphenyl derivative of retinoic acid (RO-10-9359) 10 and 25 mg capsules.

Material:

1) Keratinizing Dermatoses

   A Ichthyosis
      a) lamellar ichthyosis
      b) X-linked ichthyosis
      c) ichthyosis vulgaris
      d) epidermolytic hyperkeratosis
      e) non-bullous congenital ichthyosiform erythroderma
   B) Darier's disease
   C) Psoriasis
   D) Keratoderma palmaris et plantaris
   E) Pityriasis rubra pilaris
   F) Nevus comedonicus
   G) Erythrokeratodermia variabilis
   H) Netherton's syndrome
   I) Hailey-Hailey disease
   J) Lichen planus
   K) Pachyonychia congenita

2) Basal cell carcinoma

   A) Nevoid basal cell carcinoma syndrome
   B) Sunlight induced basal cell carcinomas
   C) Arsenical induced basal cell carcinomas
   D) X-ray induced carcinomas

3) Cystic and Conglobate acne and hidradenitis suppuritiva

Methods Employed:

1) Disorders of Keratinization
   a) Oral 13-cis retinoic acid will be given for 4 months beginning at 1 mg/kg/day. The dose will be gradually increased (up to 8 mg/kg/day to tolerance.

b) Oral RO-10-9359 will be given for 4 months beginning at 0.5 mg/kg/day. The dose will be increased up to 1.5 mg/kg/day or to tolerance.

2) Skin Cancer
a) Patients must have pathologic confirmation of the clinical diagnosis.
b) They must have normal hematologic, hepatic, and renal function.
c) Lesions on eyelids and canthi are not included.
d) Patients are cautioned to avoid sun.
d) Laboratory tests are done before, during and 6 weeks after therapy.
f) Serial photographs are taken.

3) Cystic acne
a) Patients must be 16 years of age
b) Patients must have at least 10 cystic lesions.

Major Findings:

1) Eighty-one patients with Darier's disease (DD), lamellar ichthyosis (LI), pityriasis rubra pilaris (PRP), and other keratinizing dermatoses, cystic acne, and basal cell carcinoma were treated with an oral synthetic retinoid (13-cis-retinoic acid, RO 4-3780, Hoffman-La Roche, N.J.). The patients ranged in age from 4 to 82 years. The dosage varied from 0.5 to 8.0 mg/kg/day, and the duration of treatment varied from 1 to 55 weeks. The median dosage was 160 mg or 2 mg/kg daily for 19 weeks. To date, a good to excellent response has been observed in 8 of 9 patients with LI, 3 of 3 with non-bullous congenital ichthyosiform erythroderma, (which we distinguish from LI), 1 of 1 with ichthyosis vulgaris, 8 of 9 with DD, 2 of 5 with PRP, 2 of 8 with psoriasis, and 14 of 14 with cystic acne. Partial responses were observed in epidermolytic hyperkeratosis (4 patients), PRP (2), psoriasis (1), LI (1), DD (1), and keratosis palmaris et plantaris (1). Three patients with psoriasis, 2 with X-linked ichthyosis, and one with nevus comedonicus failed to improve. One patiet with X-linked ichthyosis and one with Netherton's syndrome worsened during therapy. Of the 14 patients with cystic or conglobate acne who completed a 4 month course of therapy with 13-cis-retinoic acid, 12 had completed a 4 month course of therapy with 13-cis-retinoic acid, 12 had complete resolution (100%) of all cystic lesions, one had an 82% response, and one had a 75% response. Thus far, all 14 acne patients have had prolonged remissions with the longest follow-up after discontinuation of therapy being 14 months. Commonly observed side effects included cheilitis, conjunctivitis, facial dermatitis, xerosis, rhinitis sicca with nosebleed, and skin fragility. Unusual side effects were itching, headache, appetite changes, finger tip peeling, and inflammation of the urethral meatus. Rare side effects were hair thinning (4 patients), dryness of the mouth with a feeling of thirst (1) and a probable allergic reaction in one patient to the parabens contained in the

capsule. Laboratory abnormalities were limited to an elevated erythrocyte sedimentation rate in 13 patients (an increase of greater than 20 mm/hr over baseline), and a temporary, slight increase in SGPT in 6 patients, and SGOT in 5, and alkaline phosphatase in 2. The mechanism by which this synthetic retinoid alters these disease states is not known but may be related to the observed ability of vitamin A to affect glycoprotein synthesis and epithelial differentiation. Our results indicate that synthetic retinoids, such as 13-cis-retinoic acid, may represent a potent new class of drugs in the treatment of cutaneous disease. These diseases may also serve as useful screens in the further development of the synthetic retinoids, which could be beneficial in the prevention and treatment of epithelial neoplasia.

2) 48 patients with cutaneous disorders of keratinization are being treated with RO-10-9359, an ethyl ester of a trimethylmethoxyphenyl derivative of retinoic acid. The diseases include: Darier's disease (17), Psoriasis (9), lamellar ichthyosis (5), epidermolytic hyperkeratosis (3), pityriasis rubra pilaris (5), generalized erythroderma (3), and one each of non-bullous congenital ichthyosiform erythroderma, erythrokeratoderma variabilis, ichthyosis vulgaris, Hailey-Hailey Disease, keratosis palmaris et plantaris, and pachyonchia congenita. Responses have been uniformly good to excellent in all diseases except psoriasis and Hailey-Hailey Disease. Dosage was begun at 0.5 mg/kg/day in all patients and increased in some to 1.0 and 1.5 mg/kg/day for a maximum treatment time of 16 weeks. The response to RO-10-9359 has been superior to 13-cis-retinoic acid, especially in epidermolytic hyperkeratosis. Toxicities have been similar to those seen with 13-cis-retinoic acid, but less severe. Three patients have had temporary, low-grade elevations of SGOT and SGPT.

3) An ongoing study comparing 13-cis-retinoic acid at an initial dose of 0.5 mg/kg/day versus placebo is being done in a double-blind format. Thus far, 31 patients have been entered and 28 have had followup visits. Thirteen patients who initially received placebo worsened to the point where the double-blind code was broken and treatment with 13-cis-retinoic acid was begun with subsequent improvement in the 6 patients who had follow-up visits. Twelve of 14 patients who we suspect are receiving 13-cis-retinoic acid, due to the presence of characteristic side effects are improving significantly. One patient who we suspect to be on placebo has improved slightly. The results to date indicate that the beneficial therapeutic response is not a placebo effect, that 0.5 mg/kg/day is an effective dose, particularly for the facial lesions, which is also productive of the common side effects, and that perhaps lower doses may also be effective. Higher doses are required in several patients for control of chest and back lesions.

4) Ten patients with multiple basal cell carcinomas induced by sunlight, X-ray, arsenic, or the nevoid basal cell carcinoma syndrome

were treated with oral 13-cis-retinoic acid. Maximum dosage varied from 3.1 to 8.2 mg/kg/day (ave. = 5.5) or 240 to 660 mg/day (ave. = 400). Duration of treatment varied from 11 to 31 weeks (ave. = 20). Patients (7M, 3F) ranged in age from 29 to 57 years (ave. = 44). Three of the nevoid basal cell carcinoma syndrome patients and one of the sunlight induced BCCA patients had been sensitized to and treated with topical dinitrochlorobenzene (DNCB) six years ago. Of interest, is that 3 of these 4 patients developed a marked inflammatory response in most tumors, especially those on the head and neck, and spared the intervening, apparently uninvolved skin. In these patients when the inflammatory reaction had subsided after discontinuation of the drug an objective reduction in tumor mass was noted in 80 of 110 tumors with complete clinical regression in 9. Most of these latter tumors were originally only 3-4 mm in diameter and located on the head and neck.

The remaining 6 patients who had never been exposed to DNCB failed to develop inflammation in their tumors. Instead, a steady gradual reduction in tumor size was noted. Final results in this group were comparable to the DNCB sensitive patients with 83 of 109 tumors decreasing in size and 12 showing complete clinical regression. Histologically, a dense small cell infiltrate rich in plasma cells was seen in those tumors undergoing inflammation. In non-inflammatory, regressing tumors, islands of squamous differentiation with horn pearl formation were observed with progression in some islands to the complete replacement of tumor cells by the completely differentiated horn pearl. In those tumors showing complete clinical regression approximately 1/3 to 1/2 had negative post-treatment biopsies.

Significance to Cancer Research:

a) These are exploratory studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer. Skin cancer should prove to be a valuable lesion for the screening of these agents because the tumors are observable, measurable, and therefore can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

b) The profound beneficial effect of 13-cis retinoic acid in the treatment of acne and cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelia, could be successfully treated with the synthetic retinoids. Treatment of these keratinizing dermatoses and acne may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Proposed Course of Project:

1) Continued treatment of patients with acne, basal cell carcinomas, and disorders of keratinization with 13-cis retinoic acid (RO-43780).

2) Treatment of disorders of keratinization with oral RO-10-9359 and topical RO-11-1430).

Publications:

1) Peck, G.L. and Yoder, F.W.: Treatment of disorders of keratinization with an oral stereoisomer of retinoic acid, in The Ichthyoses (R. Marks, editor) MTP Press, Ltd., Lancaster, England, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03663-02 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Tumor virus expression in vitro and in vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Lowy	Senior Investigator	Derm NCI

COOPERATING UNITS (if any)

Laboratory of Tumor Virus Genetics

LAB/BRANCH
Dermatology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a mink cell line that contains the Moloney murine sarcoma viral genome, glucocorticoids induce foci of morphologically transformed cells and increases in viral RNA and viral p 30 antigen. Removal of glucocorticoid is followed by a return to be the base line of all parameters. In the differentiated state, mouse teratoma cells were permissive for murine leukemia viral expression; cells in the undifferentiated state showed a post penetration block to viral expression. A novel species of mouse 30S RNA has been detected in several mouse cell lines and appears to represent defective endogenous mouse C-type retroviral genetic material. The Friend strain of spleen focus-forming virus was shown to be a recombinant between ecotropic murine type-C retrovirus and the env gene region of xenotropic type-C retrovirus. Unintegrated Harvey sarcoma viral DNA, a linear molecule of 4×10^6 daltons, can transform cells without helper virus or its proteins. DNA from AKR cells which contain unexpressed endogenous leukemia viral genomes is not infectious unless the recipient cells are treated with 5-iododeoxyuridine.

Project Description:

Objectives:

1) To evaluate the effect of cell differentiation, primarily by chemical or hormonal treatment, on retrovirus expression and transformation in cells in tissue culture.

2) To evaluate the effects on hormones and other chemicals on endogenous and exogenous virus expression.

3) To gain insight into how the state of differentiation of a cell regulates tumor virus expression.

4) To study viral recombination, especially as it relates to the development of sarcoma viruses.

5) To develop a sensitive quantitative biologic assay of viral-specific DNA (transfection) from C-type viruses.

6) To determine the effect of a variety of physical and chemical treatments of the transfecting ability of viral-specific DNA.

7) To determine the physical differences between unexpressed and expressed endogenous DNA, since transfection of expressed viral DNA is more easily accomplished than that of unexpressed viral DNA.

8) To define the smallest size of cellular DNA which can yield infectious virus and/or biologic activity.

9) To investigate the mechanism of activation of endogenous C-type viruses by halogenated pyrimidines.

10) To determine if transfection by the DNA viral intermediate and/or the DNA provirus is sensitive to the murine cellular Fv-1 gene, which restricts the replication of some classes of murine C-type viruses.

11) To define that the portion of the sarcoma virus genome which induces cellular transformation.

12) To utilize transfection as a method to detect portions of viral genomes in tumors, including those of human origin.

13) To develop a radioimmune assay for wart viral protein(s).

14) To develop nucleic acid hybridization procesures for wart viral DNA.

15) To propagate wart viruses in tissue culture.

16) To measure the relatedness of viruses of warts from different

patients, including those from patients with epidermodysplasia verruciformis (EDV), laryngeal papillomas, condyloma accuminata, flat warts, common warts, plantar warts, and warts associated with immunodeficiency.

17) To look for wart virus specific nucleic acid and/or antigen in skin cancers of EDV, kerato-acanthomas, and other tumors.

18) Relatedness of wart viruses to other papova viruses.

Methods:

1) Treatment of cells or animals with hormones, other chemicals, and tumor viruses.

2) Detection of retrovirus expression biologically by XC plaque test and focus induction.

3) Radioimmune assays for viral antigens.

4) For the isolation of provial genomes, DNA is extracted from tissue culture cells or tumors by the Marmur technique, except that proteinase K is used instead of pronase, since higher molecular weight DNA is obtained by this modification.

5) For isolation of the viral DNA intermediate, DNA is purified by the Hirt procedure.

6) Transfection of DNA utilizes the calcium phosphate technique of Graham and Van der Eb as modified by Stow and Wilkie.

7) Virus is grown in sensitive tissue culture cells and purified by sucrose density centrifugation in a zonal rotor. Isotopically labeled single stranded viral DNA probes are synthesized in an endogenous reverse transcriptase reaction carried out in the presence of actinomycin D or in exogenous reactions following purification of viral RNA on sucrose density gradients.

8) Nucleic acid hybridization between viral probes and cell DNA is carried out in liquid and analyzed by S_1 nuclease or hybrization is performed using the Southern Technique of transferring DNA from agarose gels to nitrocellulose filters.

9) Infectious DNA is treated with restriction endonucleases and assayed for residual biologic activity. This activity is then correlated with the cleavage pattern of viral DNA.

Results:

1) An untransformed cloned mink cell line which contains the Moloney

murine sarcoma viral (MSV) genome will undergo focus induction on treatment with physiologic concentrations of dexamethasone or other glucocorticoids. Focus induction correlated with the levels of sarcoma specific viral RNA and with viral p30 protein. Transformation depends on the continued presence of glucocorticoid, since its removal results in loss of transformation and a decrease in viral expression to pretreatment levels. Some cells apparently lack the MSV genome, since neither focus induction nor a rescuable sarcoma genome was found in some sub-clones.

2) In collaboration with Dr. N. Teich (Imperial Cancer Research Fund, London), several mouse teratoma cell lines have been examined for their ability to be infected by a variety of viruses. In the undifferentiated state, these cells were permissive for the replication of all viruses tested except for murine leukemia viruses. After differentiation, a cell line became permissive for Moloney leukemia virus. Nucleic acid hybridization studies indicate that the block to viral expression occurs after synthesis of proviral DNA, since as much viral DNA is found in the non-productively infected undifferentiated cells as in the level of transcription since viral RNA was not detected in the undifferentiated cells.

3) The spleen focus-forming virus (SFFV) has acquired specific xenotropic viral genetic information not contained in Friend helper virus. A cDNA probe that represents the xenotropic virus portion of SFFV detects genetic sequences derived from the env gene region of murine xenotropic virus. SFFV therefore represents a recombinant between ecotropic and xenotropic viral genetic information.

4) A novel species of 30S RNA has been detected in a variety of mouse cell lines. The 30S RNA is specifically packaged by helper independent type-C viruses propagated in these cells. Nucleic acid hybridization detects no homology between the 30S RNA and the genomic RNA of helper independent mouse type-C viruses. These results suggest that the 30S RNA is a defective endogenous mouse type-C retrovirus.

5) A sensitive, quantitative 10 day transfection assay for murine leukemia and murine sarcoma viruses has been developed for use with unintegrated and integrated viral DNA.

6) Infectious unintegrated Moloney murine leukemia virus (Mo-MuLV) and Harvey sarcoma virus (Ha-SV) DNA titrate with single hit kinetics in the transfection procedure. Mo-MuLV and Ha-SV infectious molecules can be separated from each other on agarose gels, in which they migrate as linear double-stranded DNAs. The MoMULV has a molecular weight of 6×10^6 daltons while the Ha-SV DNA has a molecular weight of 4×10^6 daltons. Infectious DNAs therefore have approximately the same number of bases as a single unit of viral RNA. Treatment of the DNA with restriction endonuclease Eco R1 cleaves the Ha-SV DNA at one site and abolishes its transforming activity while the Mo-MuLV DNA, which does

not contain an Eco R1 site, remains infectious. Conversely, treatment of the DNA with restriction endonuclease Xho I cleaves the Mo-MuLV DNA at one site and abolishes its infectivity while the Ha-SV DNA, which does not contain an Xho I site, still transforms cells.

7) Transfection of infectious integrated retroviral DNA from cells which are chronically infected with AKR MuLV can be accomplished with the transfection system. Under similar conditions, however, cellular DNA from the AKR mouse, which is known to contain the endogenous AKR MuLV genome but in an unexpressed form, is not infectious. The transfecting AKR DNA can, however, become infectious if the recipient cells are treated with 5-iododeoxyuridine, an agent known to activate AKR MuLV genomes in AKR cells in tissue culture.

Significance to Cancer Research:

1) C-type viruses have been implicated in tumors of a wide variety of species, including man. Correlation of biologic and biochemical aspects of viral specific DNA will be of considerable help in elucidating the physiology of mammalian C-type viruses. The above studies show that the size of transforming Ha-SV DNA has been established. In addition, unintegrated Ha-SV DNA can transform cells in the absence of helper viral proteins. The integrity of the viral genome appears to be required for infectivity to be maintained. The ability to separate infectious sarcoma viral DNA from leukemia viral DNA, either on the basis of size, by agarose gel electrophoresis, or by different restriction endonuclease sites, provides two methods which for the first time permit infectious mammalian sarcoma viral molecules to be studied independently of leukemia viral molecules.

2) The efficiency of transfection of integrated and endogenous viral genomes correlates with their expression or lack thereof in the cells from which the DNA has been extracted. However, the restriction to transfection of the endogenous AKR MuLV genome can be abolished by treatment of recipient cells with a compound known to activate AKR MuLV from AKR cells. These results imply that the arrangement of the viral DNA in endogenous and integrated viral genomes is physically different. Elucidation of these differences may establish fundamental mechanisms by which cells control expression of viral genomes.

3) The ability of hormonal manipulation to affect tumor development has been well documented both in man and animals. Under certain conditions, hormones may also affect tumor virus expression. The $S^{+}L$-mink system represents the first hormone responsive system where focus induction appears to depend on retrovirus expression. It should permit probing of the mechanisms by which steroid hormones might affect the development of cancer.

4) The issue of how cells control gene expression is central to the problem of the cancer cell. The teratomas provide a system where virus

replication is clearly regulated by a cellular function whose expression depends on the differentiated state of the cell. Exploration of this phenomenon may provide insight into how mammalian cells regulate the expression of genetic material.

5) Recombination occurs frequently amont type-C retroviruses and appears to represent an important pathogenetic event in the development of viral induced tumors. Mammalian sarcoma viruses represent recombinants between leukemia viral sequences and endogenous cellular sequences. In the rat, a 30S RNA is detected in numerous cell lines; these cellular sequences appear to represent defective C-type retroviral genomes and are represented in the rat portion of Harvey sarcoma virus and Kirsten sarcoma virus. It now appears that the Friend spleen focus-forming virus, a replication-defective virus which causes the rapid transformation of certain hematopoietic target cells, is a recombinant virus, analogous to the classic mammalian sarcoma viruses. Detection of the 30S RNA in the mouse represents a second species in which a cellular RNA is specifically packagable by type-C retroviruses.

Proposed Course of Project:

Continuation with attempted fullfillment of stated objectives.

Publications:

1. Troxler, D.H., Lowy, D., Howk, R.S., Young, H., and Scolnick, E.M.: The spleen focus-forming virus is a recombinant between ecotropic murine type-C virus, and the env gene region of xenotropic type-C virus. Proc. Nat. Acad. Sci. USA 74:4671, 1977.

2. Teich, N.M., Weiss, R.A., Martin, G.R., and Lowy, D.R.: Virus infection of murine teratocarcinoma stem cell lines. Cell 12:973, 1977.

3. Lowy, D.R., and Scolnick, E.M.: Glucocorticoids induce focus formation and increase sarcoma viral expression in a mink cell line that contains a murine sarcoma viral genome. J. Virol. 25:157, 1978.

4. Howk, R.S., Troxler, D.H., Lowy, D.R., Duesberg, P.H., and Scolnick, E.M.: Identification of a 30S RNA with properties of a defective type-C virus in murine cells. J. Virol. 25:115, 1978.

5. Lowy, D.R., Rands, E., and Scolnick, E.M.: Helper independent transformation by unintegrated Harvey sarcoma virus DNA. J. Virol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03664-01 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cellular Interactions in the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Hernandez	Visiting Medical Researcher	Derm NCI
OTHER:	J.E. Bennett	Senior Investigator	NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigations in strain 13 guinea pigs have revealed a population of cells capable of expressing suppression and augmentation of T-cell function. Characterization and purification of the suppressor cells and helper cells are under investigation.

Project Description:

Objectives:

1) To study the interaction of cells from different anatomical compartments which result in a positive (helper) or negative (suppressor) regulation of T-cell function.

2) To identify, characterize and purify populations of cells which act as helpers or suppressors of T-cell function.

Methods:

1) Strain 13 guinea pigs, unsensitized and sensitized with CFA for 2 and 4 weeks were used as donors. Mononuclear cells were obtained from cardiac blood, lymph nodes and spleens.

2) Mononuclear cells from these sites were cultured in vitro separately or in equal combination with mononuclear cells from one other site. Cellular function was assayed using lymphocyte transformation stimulated by T-cell mitogen.

3) Characterization of the subpopulations of mononuclear cells responsible for the observed interaction of mononuclear cells from different anatomical sites was investigated using:

a) Passage through Sephadex G-10 columns
b) E-Rosetting with rabbit erythrocytes
c) 1000 R X-irradiation
d) Mitomycin C treatment
e) Fluorescent Ab tagging

Major Findings:

I. When lymphocyte transformation stimulated by a T-cell mitogen was used as an indicator of T-cell function, the following were observed:

1) When cardiac blood and lymph node cells were obtained from an unsensitized animal, the interaction of these two populations was greater than the sum of each assayed separately (Helper effect).

2) When cardiac blood and lymph node cells were obtained from a two week-post CFA animal the interaction of the two populations was essentially identical to the sum of each assayed separately (no helper or suppressor effect).

3) When cardiac blood and lymph nodes cells were obtained from 4 week-post CFA animals, the interaction of these two populations was less than the sum of each assayed separately (suppressor

effect). The interaction of spleen and lymph node cells from these animals in contrast, showed a helper effect.

II. The suppressive effects of the interaction of cardiac blood cells and lymph node cells were ablated by modifying the cardiac blood cells with 100 R X-irradiation, mitomycin C treatment, or using non-E-Rosetted cells. Passage of the cardiac blood cells through sephadex G-10 or using E-rosetted cells did not change the suppressor effects. Thus a suppressor function reside in a population of cells which have characteristics of T-cells.

Significance to Cancer Research:

The understanding of the populations of cells and the mechanisms by which they play a role in the modulation of the immune response, may have direct implications on the pathogenesis and treatment of patients with malignancies.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03665-01 D

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Epidermodysplasia Verruciformis (EV)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.A. Lutzner	Chief	Derm, NCI
OTHER:	M. Prunieras	Chief	Laboratory for Skin Tumor Research, Paris
	G. Orth	Virologist	Villejuif, Paris
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COOPERATING UNITS (if any)

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LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Case reports of patients with EV have been reviewed. It has been determined that EV is an autosomal recessive disease, accompanied in at least 10% of cases by mental retardation. Flat warts occur in all patients, pityriasis-like warts in 75% of cases. Cancer of the skin develops in 1/3 of cases. Relationship of mutant gene to viral oncogenesis and mental retardation is unknown.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To review the world's literature on EV. To collect tissue from these patients for viral typing and viral studies. To grow cells from these patients for chemical and biological testing. To do psychometric testing in an attempt to identify the type of retardation. To collect tumor tissue for viral studies. To attempt to identify the gene defect.

Materials:

200 + cases of EV reported in literature. Gene survey, clinical examination. Skin biopsies and serum from EV patients.

Methods Employed:

Genetic analysis of EV case histories. Culturing fibroblasts for chemical studies and karyotype analysis. Virus studies-immunological and chemical. Immunological study of patients. Study of type of mental retardation by psychological testing.

Major Findings:

1) Using consanguinity index and sibling data, EV is a disease with autosomal recessive inheritance.

2) Mental retardation is a bonafide concomitant of EV, occuring in at least 10% of cases.

3) Pityriasis - like warts (a type of wart which probably appears only in EV patients) are found in about 75% of patients.

4) It has been found by Orth and Jablonska that type III wart virus is associated with flat warts and type IV virus is associated with pityriasis - like warts.

Significance to Cancer Research:

Warts occur in almost all children at one time or another, but is only rarely associated with skin cancer. EV patients have chronic, widespread warts and 1/3 of patients develop skin cancer. Wart virus/cancer relationships in these patients should be explainable by a single mutant gene, which information should provide new ideas about viral oncogenesis. The relationship of mental retardation to oncogenesis is problematic, but explanation of the phenomenon might provide new insight as to cognitive functions.

Publications:

None

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